Molecular Mechanisms of Inflammation-Induced Cancer: T-cells as Targets for Prevention and Cure of *Helicobacter*-Related Gastric Preneoplasia

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde (Dr.sc.nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2010

Summary

Gastric adenocarcinoma is one of the most common causes of cancer-related deaths worldwide and develops as a consequence of chronic gastric inflammation caused by persistent infection with the bacterium *Helicobacter pylori*. In humans as well as in experimentally infected mice, gastric carcinogenesis progresses through a series of preneoplastic lesions which manifest histologically as chronic atrophic gastritis, epithelial hyperplasia, intestinal metaplasia and dysplasia. Gastric tumorigenesis is preceded by excessive local and systemic immune responses against *Helicobacter pylori*. It has become increasingly obvious that host genetic predisposition combined with the virulence properties of the colonizing *Helicobacter* strain determine the severity of disease outcome. Bacterial eradication shows beneficial effects in a subset of patients with atrophic gastritis, but fails to reverse intestinal metaplasia and to prevent malignant transformation. We and others have shown recently that *Helicobacter*-induced stomach-infiltrating pathogenic $CD4^+$ T-effector cells and their cytokines constitute central mediators of gastric preneoplasia.

Therefore, the aim of this study was to identify therapeutic targets that modulate the *Helicobacter*-specific T-cell response and thus can be exploited to prevent the progression of *Helicobacter*-induced premalignant lesions. We utilized a C57BL/6 mouse model of experimental *Helicobacter felis* infection in which the development of gastric preneoplasia is reminiscent of the human setting. In this model, infected mice develop chronic atrophic gastritis accompanied by epithelial hyperplasia and intestinal metaplasia as early as three months post infection. I chose to focus on two molecular players known to play crucial roles at the interface of inflammation and cancer, the cyclooxygenases (COX) and poly(ADP-ribose) polymerases (PARPs). Both enzymes are obvious candidates to assess in the context of *Helicobacter*-induced gastric preneoplasia for the following reasons:

COX-2 and its main product PGE_2 function as potent immunosuppressants and modulators of inflammatory responses on the one hand, and possess tumor promoting properties on the other. The process catalyzed by PARPs, the poly(ADP-ribosyl)ation of specific acceptor proteins, is an important post-transcriptional modification involved in the regulation of pro-inflammatory genes, and also participates in the DNA damage response after genotoxic damage. We utilized pharmacological as well as genetic approaches to manipulate these key players at the interface of inflammation and cancer and to elucidate their contribution to *Helicobacter*-induced preneoplasia.

The pharmacological inhibition of COX-2 enzymatic activity during *Helicobacter* infection in mice led to significantly accelerated and aggravated disease symptoms characterized by more severe gastritis and epithelial pathology as well as elevated IFN-γ levels. In line with these findings, the administration of synthetic PGE_2 to infected mice completely suppressed and even reversed pre-existing *Helicobacter*-associated

precancerous lesions, including intestinal metaplasia. The protective effect of PGE_2 treatment was accompanied by decreased gastric IFN-γ levels, reduced gastric neutrophil and T-cell infiltration and a reduction of T-cellular Th-1 differentation in the draining mesenteric lymph nodes. Similarly, systemic administration of the inhibitor of poly(ADP-ribosyl)ation, PJ34, reversed pre-existing metaplastic lesions and provided sustainable cure if the treatment was combined with bacterial eradication therapy. The protective effect of both treatments was found to be due to the potent suppression of pathogenic T-cell responses. Data obtained from complementary *ex vivo* and *in vitro* experiments indicate that PGE_2 and PJ34 treatments abolish T-cellular proliferation, activation-dependent cytokine secretion and migration of $CD4^+$ T-effector cells. This effect was accompanied and probably caused by the transcriptional suppression of the genes encoding IFN-γ and IL-2, the latter being a cytokine essential for continuous effector and regulatory T-cell stimulation and perpetuation of adaptive immune responses.

Another focus of this study was to elucidate *Helicobacter pylori*'s intrinsic genotoxic propterties and to thus determine a possible direct contribution of the bacteria to gastric carcinogenesis. We investigated the potential of *Helicobacter pylori* to directly damage host cellular DNA and thereby contribute to the acquisition of tumor-promoting mutations. To this end, various cell lines and primary cells were infected with *H. pylori* and the induction of DNA double strand breaks (DSB) and subsequent DNA damage signalling was investigated. Pulse field gel electrophoresis revealed that *H. pylori* infection causes DSB in an infection dose-dependent manner. The appearance of DSB depended on contact of live bacteria with the host cell, but occurred independently of the *H. pylori* virulence factors VacA and the Cag pathogenicity island and was not observed upon infection with *E. coli*. *H. pylori* infection-induced DSB resulted in a DNA damage response evident as phosphorylation of H2AX (γH2AX) and foci formation of 53BP1 and MDC1 proteins at sites of DSB. DSB were efficiently repaired by the host cells, but only after antibiotic eradication of *H. pylori*. The data suggest an active role of *Helicobacter* in the process of host cellular transformation, and may involve a specific bacterial factor delivered upon host-pathogen contact.

Taken together, the data obtained in the course of my PhD thesis show that *Helicobacter* possesses genotoxic properties and that pharmacological targeting of $CD4^+$ T-cells inhibits and reverses pre-existing precancerous lesions in a mouse model of *Helicobacter*-induced gastric preneoplasia. The results thus provide insights into new therapeutic targets for the treatment of patients that are refractory to *Helicobacter* eradication therapy or present with untreatable gastric cancer precursor lesions.

Zusammenfassung

Das Magenkarzinom zählt zu den häufigsten tödlichen Krebserkrankungen weltweit. Die Entstehung basiert auf einer chronischen Entzündung der Magenmukosa, verursacht durch eine persistierende Infektion mit *Helicobacter pylori*. Bei Menschen wie bei Mäusen verläuft die gastrale Karzinogenese ausgehend von präneoplastischen Krebsvorstufen. Histologisch manifestieren sich diese Vorstufen als atrophische Gastritits, epitheliale Hyperplasie, intestinale Metaplasie und Dysplasie. Die gastrale Krebsentstehung ist somit ein immunpathologischer Prozess, welcher durch eine übertrieben starke Immunantwort gegen das magenkolonisierende Bakterium *Helicobacter pylori* verursacht wird. Die Virulenz des *Helicobacter*-Stammes sowie die genetische Prädisposition des Wirtes bestimmen den Schweregrad der fortschreitenden Erkrankung. Bei Personen mit bestimmten gastralen Präkanzerosen kann durch die Eradikation des Keimes ein Rückgang der Erkrankung herbeigeführt werden. Intestinale Metaplasien und deren Weiterentwicklung zu einer malignen Erkrankung hingegen können dadurch nicht verhindert werden. Daten, die in unserm Labor generiert wurden zeigen, dass pathogene T-Zellen und deren Zytokine für die Entstehung von Magenkrebs und dessen Vorstufen mitverantwortlich sind.

Das Ziel der vorliegenden Arbeit war es, mögliche verantwortliche Faktoren und gegen dieselben gerichtete Wirkstoffe zu identifizieren, welche pathogene T-Zell Antworten hemmen und dadurch das Fortschreiten von gutartigen Krebsvorstufen zu bösartigen Läsionen unterbinden können. Zudem untersuchten wir, ob *H. pylori* selbst die DNS der infizierten Wirtszellen schädigen und somit selbst kanzerogen wirken kann.

Für die Studien infizierten wir C57BL/6 Mäuse mit *Helicobacter felis*, einem nahen Verwandten des human-pathogenen *H. pylori.* Die Entstehung der Präkanzerosen in unserem Modell ist mit derjenigen im Menschen vergleichbar. Infizierte Mäuse entwickeln nach drei Monaten verschiedene Krebsvorstufen in der Magenmukosa: Gastritis, Hyperplasie des Epithels und intestinale Metaplasie. Wir untersuchten die Funktion von zwei wichtigen Komponenten, die einerseits während der Entzündungsreaktion und andererseits auch in bestehenden Tumoren eine wichtige Rolle spielen und somit als Beschleuniger der Krebsentwicklung fungieren könnten: die Cyclooxygenasen (insbesondere COX-2) und die Poly(ADP-ribose)polymerasen (PARPs). Das von der COX-2 synthetisierte PGE_2 hemmt die Immunantwort, koordiniert die Entzündungsreaktion und besitzt andererseits aber auch krebsunterstützende Funktionen. Eine ähnlich wichtige Rolle spielt die post-transkriptionelle Poly(ADP-ribosyl)ierung von Proteinen, die in der Entzündungsreaktion mitwirken. Den Beitrag dieser Proteine zur *Helicobacter*-induzierten Krebsentstehung untersuchten wir, indem wir die Funktion der beiden Vorgänge pharmakologisch unterdrückten.

Eine Blockierung der COX-2 und folglich auch der PGE_2 Synthese während der Infektion führte zu einem signifikant beschleunigten Krankheitsverlauf und zu verstärkten

Krankheitsmerkmalen im Bereich der Magenmukosa der infizierten Mäuse. Zusätzlich konnten wir hohe IFN-γ Konzentrationen und eine geringere Anzahl Bakterien in der Magenmukosa nachweisen. Übereinstimmend mit dieser Beobachtung bewirkte eine Verabreichung von exogenem PGE_2 eine markante Regredienz der Gastritis und unterdrückte nicht nur die Entstehung von *Helicobacter*-assoziierten Präkanzerosen, sondern bewirkte auch einen Rückgang der intestinalen Metaplasie. Dies ging mit tiefen IFN-γ Konzentrationen und hoher bakterieller Kolonisierung im Magen einher.

Zusammenfassend zeigen die erhobenen Daten, dass die Verabreichung von exogenem, synthetischen PGE_2 an infizierte Mäuse nicht nur die Entstehung von Präkanzerosen unterdrückte, sondern es zeigt sich auch, dass bestehende präkanzeröse Läsionen einschliesslich der intestinalen Metaplasien geheilt werden konnten und die Magenschleimhaut sich in der Folge normalisierte. Diese Resultate sprechen für eine immunsuppressive Rolle von PGE_2, was dazu führt, dass keine Immunzellen in die Magenschleimhaut einwandern können um die *Helicobacter*-Infektion zu bekämpfen, was wiederum eine hohe *Helicobacter*-Kolonisierung zur Folge hat.

Einen ähnlichen Effekt zeigte die Behandlung von infizierten Mäusen mit PJ34. In behandelten Mäusen konnte ebenfalls die Entstehung von *Helicobacter*-induzierten Krebsvorstufen verhindert werden und auch bereits bestehende Veränderungen der Magenschleimhaut konnten geheilt werden.

Unsere *in vitro* Untersuchungen haben weiterhin gezeigt, dass eine Behandlung mit PGE_2 und PJ34 die Aktivierung von T-Zellen hemmt und damit die entzündungsinduzierte Zytokinsekretion und die Migration von $CD4^+$ Effektor-T-Zellen zum Infektfokus verhindert. Zudem führte die Behandlung zu einer Blockierung der Transkription von IFN-γ und IL-2. IFN-γ spielt eine wichtige Rolle bei der Bekämpfung der Kolonisierung, führt aber gleichzeitig auch zur Entstehung von Veränderungen in der Magenschleimhaut. IL-2 ist ein essentielles Zytokin, das für die Aktivierung und die Proliferation von regulatorischen und Effektor-T-Zellen sowie für die Aufrechterhaltung der erworbenen Immunantwort verantwortlich ist. Dies erklärt auch den lindernden Effekt von PGE_2 und PJ34. Mit andern Worten, die beiden Substanzen verhindern die Entstehung einer Entzündungsreaktion im Magen, indem sie Proliferation und die Sekretion von Effektorzytokinen durch T-Zellen unterdrücken.

Neben den immunpathologischen Ursachen der gastralen Karzinogenese untersuchten wir auch, ob *Helicobacter pylori* die DNS der Wirtszelle direkt schädigen kann und dadurch die Tumorentstehung provozieren könnte. Dazu infizierten wir verschiedene Zelllinien mit *H. pylori* und untersuchten dann die Intaktheit der Wirts-DNS und ob dabei DNS-Doppelstrangbrüche (DSB) entstanden sind. Ausserdem untersuchten wir, ob die betroffenen Zellen auf die DNA Schäden mit der Aktivierung assozierter Signalkaskaden reagierten. Wir konnten feststellen, dass die *Helicobacter*-Infektion tatsächlich DNS Schäden

induziert. Darüber hinaus haben wir gezeigt, dass die DSB durch wirtseigene Reparaturmechanismen repariert werden konnten, aber nur nachdem die Bakterien vorher abgetötet worden waren.

Die Voraussetzung für die Entstehung von DSB war der Kontakt der lebenden Bakterien mit den Zellen, wobei die DSB aber unabhängig von den bekannten *H. pylori*-Virulenzfaktoren VacA und CagPAI hervorgerufen wurden. Des weiteren induzierte die bakterielle Infektion DSB-abhängige Signalkaskaden wie die Phosphorylierung von H2AX (γH2AX) und die Fokusbildung der 53BP1 und MDC1 Proteine. Unsere Daten deuten darauf hin, dass *H. pylori* die Wirts-DNA direkt schädigt, also auch ohne Einwirkung von Entzündungszellen und so möglicherweise eine Wirtszellentransformation bewirken oder fördern kann.

Zusammengefasst zeigen unsere Resultate also auf, dass *H. pylori* genotoxisches Potenzial besitzt und dass spezifische Therapien, welche die pathogene T-Zell-Immunantwort hemmen nicht nur die von *H. pylori* induzierten Präkanzerosen unterdrückten, sondern dass bereits bestehende Läsionen sich zurückbilden konnten. Diese gewonnenen Erkenntnisse dienen somit als Grundlage für die Entwicklung entsprechender Medikamente zur Behandlung von resistenten *Helicobacter*-Infektionen und Präkanzerosen, die das Entstehen eines Magenkrebses in Zukunft verhindern sollen.

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1. Introduction

1.1 Gastric Cancer

1.1.1 Gastric Cancer and *Helicobacter* Infection

Stomach cancer has been recognized for several millennia and Hippocrates' first description of a patient with melaina (black vomiting) as early as 400 BC most likely characterized cancer of the stomach.[9] Only in the last thirty years, persistent *Helicobacter pylori* (*H. pylori*) infection has been epidemiologically linked to gastric and duodenal ulcers, gastric mucosa-associated lymphoid tissue lymphoma (MALT), and gastric cancer.[10, 11] The present study will focus on cancer precursor lesions that will ultimately result in gastric adenocarcinoma. Gastric adenocarcinoma develops over decades as a consequence of chronic inflammation of the stomach lining that is caused by persistent *H. pylori* infection and belongs to the leading causes of cancer-related deaths world-wide.[12] However little is known about benign cancer pre-cursor lesions. The following section will provide an overview of the prevalence of gastric cancer, elucidate possible risk factors and describe the most important risk factor of gastric cancer, *Helicobacter pylori* infection.

1.1.2 Gastric Cancer Development in Mouse and Man

A causal relationship between *H. pylori* and gastric cancer was first postulated by Marshall and Warren after they had succeeded to isolate and cultivate the *Helicobacter* species from a gastric biopsy in 1982.[13] Two histologically distinct variants of distal (also non-cardia) gastric adenocarcinoma have been described and are commonly associated with *H. pylori* infection: the intestinal-type (well differentiated) and diffuse-type (undifferentiated) adenocarcinomas.[14] Intestinal-type gastric adenocarcinoma are structures resembling functional glands of the gastrointestinal tract and are found predominantly in areas with high incidence of gastric cancer, usually occur at a late age, predominate in men and progress through a relatively well-defined series of histological steps. Diffuse adenocarcinomas consist of individually infiltrating neoplastic cells that do not form glandular structures and are not associated with intestinal metaplasia. Furthermore, the tumor cells are no longer capable of performing basic gastric functions[14] and have been shown to commonly affect younger people, whereas men and women are equally affected.[14,15] *Helicobacter* infection significantly increases both types of cancers; this introduction will however only focus on the progression of intestinal-type adenocarcinoma, which we can model in experimentally infected mice.

In human as in mice, gastric carcinogenesis progresses through a sequence of preneoplastic lesions, also called the Correa pathway.[16] The chain of events that occurs

during the development of *Helicobacter*-associated intestinal-type adenocarcinoma involves the transition from normal mucosa to normal superficial gastritis, to atrophic gastritis (including pit cell hyperplasia) to intestinal metaplasia (=cancer precursor lesions) and finally to dysplasia and adenocarcinoma as depicted in Figure 1.[7]

C57BL/6 mice infected with *Helicobacter felis,* a close relative of *H. pylori,* develop pre-cancerous lesions of the intestinal-type reminiscent of the human setting.[17] The sequelae of gastric cancer pre-cursor lesions are characterized by a corpus-dominated superficial gastritis, atrophic gastritis with epithelial hyperplasia and intestinal metaplasia. Progression to malignant dysplasia[18] is rarely observed and observed only with additional carcinogen treatment (MNNG) or in mice with hypergastrinemia (INS-GAS).[19]

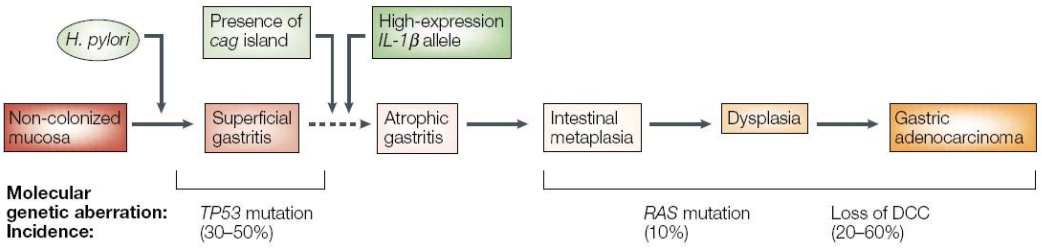

Figure 1 *Helicobacter*-induced gastritis and the progression to intestinal-type gastric adenocarcinoma. *Helicobacter pylori* colonization usually occurs during childhood and, over a period of days to weeks, leads to superficial gastritis. The presence of host *TP53* mutations, host polymorphisms that promote high expression levels of the cytokine interleukin (IL)-1β, but also TNF-α and IL-10, and the cag island within infecting *H. pylori* isolates all contribute to the development of atrophic gastritis, intestinal metaplasia, dysplasia and, eventually, gastric adenocarcinoma over the course of many years. Additional mutations in oncogenes that encode RAS or deleted in colorectal cancer (DCC), but also epigenetic modifications of MLH-1, E-cadherin and p16 might also contribute to intestinal-type gastric carcinogenesis (modified from Peek and Blaser, 2002).[7]

The risk for developing gastric cancer increases exponentially as the extent of atrophic gastritis and intestinal metaplasia increase.[15] Even though there is no defined sequence of mutations occurring in different tumor suppressor or oncogenes as it is observed in colon cancer, mutations in *TP53, RAS a*nd loss of DCC potentiate the risk for cancer progression (Figure 1).[7]

Several case-controlled studies have shown that *H. pylori* seropositivity is associated with a 2.1-16.7% fold increased risk of gastric cancer; [10, 20-26] however the actual *H. pylori* prevalence may be underestimated due to the diminished *Helicobacter* presence in premalignant lesions. The importance of *Helicobacter* infection in gastric carcinogenesis is further reflected by the correlated decrease of gastric cancer incidence and *Helicobacter* infections in Western countries[27, 28] and the beneficial effect of bacterial eradication on gastric cancer regression.[29] However, although *H. pylori* is thought to account for 80% of gastric cancers, only 15-20% of infected individuals develop more severe forms of gastric disease and only 1-2% ever develop gastric cancer, suggesting that *H. pylori* infection on its own is generally not sufficient to cause cancer.[7]

1.2 *Helicobacter* spp.

1.2.1 Prevalence and Transmission

H. pylori is a gram-negative, spiral shaped and microaerophilic bacterium that infects approximately half of the world's population, yet only a fraction of the infected population ever develop malignant disease.[30] The infection is spread via faecal-oral or oral-oral routes and can persist throughout life if not treated with antibiotics.[31] Individuals of all geographical areas may carry the bacteria, though the *Helicobacter* prevalence is higher in developing countries compared to the Western world.[32, 33] In the United States, *Helicobacter* is present in 10-15% of all children less than 12 years of age, compared to 50-60% of adults over 60 years of age. Acquisition of *Helicobacter* in adulthood is lower than 1% per year and also drops in children due to the widespread use of antibiotics.[32-34] Risk factors for *H. pylori* acquisition include low socioeconomic status, household crowding, country of origin and ethnicity.[32, 35]

1.2.2 Virulence Determinants

Distinct bacterial factors enable *H.plyori* to persist in the gastric lumen, either free-living in the gastric mucous layer or attached to gastric epithelial cells. The urease enzyme hydrolyzes urea to ammonia and carbon dioxide and thereby neutralizes the gastric acid in the bacterial periplasm; flagella allow the bacteria to move, navigate and persistently colonize the mucous layer overlying the gastric epithelium.[36] Furthermore, *Helicobacter* has evolved a variety of other virulence determinants that facilitate colonization or are associated with disease. These include the vacuolating toxin A (VacA), the *H. pylori* neutrophil-activating protein (HP-NAP) and the cag pathogenicity island (cagPAI). HP-NAP is a cytosolic 150kDa decamer able to promote neutrophil adhesion to epithelial cells[37] and mediates *H. pylori* adhesion to epithelial cells.[38] VacA is a 95-kDa, secreted protein that induces cellular vacuolization in epithelial cells and is able to modulate the host's immune response by interfering with IL-2 dependent T-cell activation[39, 40] and MHC class II dependent antigen presentation,[41] which allow it to avoid specific killing by the host's immune system.

The cagPAI is encoded by the 31 genes containing 40kb *cag* locus. A functional *cagPAI* codes for a type IV secretion system (T4SS) that allows the bacteria to translocate proteins into the host cell,[42] including the the cytotoxin-associated antigene A (CagA). Once inside the cell, CagA has been shown to deregulate the SHP2 oncoprotein among a variety of other host cell signalling pathways e.g. Ras/MEK/ERK and others.[43] Thus, infection with *H. pylori* strain harbouring the a CagA delivery-proficient CagPAI confers a higher risk for severe gastric diseases in human.[44, 45] In mouse models, however, cagA negative strains such as *H.felis,* the strain we have been using to perform the here presented studies and

close relative of *H. pylori*, appear to be as carcinogenic (or more so) compared with cag-positive *H. pylori* strains. *H.felis* in mice causes prominent chronic inflammation that is followed by atrophic gastritis, hyperplasia and metaplasia;[18, 46] most cagPAI positive *H. pylori* strains in contrast do not successfully colonize the murine host.

1.3 Host Immune Response to *Helicobacter* Infection

Despite mechanisms *H. pylori* has evolved to escape killing by the host immune response, infected individuals mount a substantial immune reaction following *H. pylori* infection. Cells of the innate immune system recognize the bacteria through receptors of so-called pathogen-associated molecular patterns (PAMPs). PAMPs include microbial components such as LPS, peptidoglycan, flagellin and double-stranded RNA that are recognized by Toll-like receptros (TLRs).[47] The initial response is characterized by neutrophil and macrophage infiltration and the release of high levels of pro-inflammatory cytokines including IL-8, IL-1β, TNF-α, and IL-6, iNOS,[48-53] and chemokines such as RANTES and MIP-1α and β.[54] The acute immune response is followed by an adaptive immune response mediated by T-lymphocytes with predominant production of the Th1 cytokines IFN-γ, IL12 and IL18,[55, 56] to a lesser extent Th2 cytokines and activation of B-cell mediated humoral responses. Despite the massive immunological response, humans as well as mice fail to clear the infection and subsequently, chronic inflammation persists plays an important role as a potential risk factor to initiate gastric carcinogenesis.[44]

1.4 Chronic Inflammation and Cancer Risk

Clinical and epidemiological studies suggest a strong correlation between chronic infection (with viruses, bacteria etc.), chronic inflammation and cancer development[36, 57, 58] and it there is no doubt that cigarette smoking[59, 60] and asbestos,[61, 62] exposure cause inflammation of the lung leading to lung cancer; alcohol abusus causes inflammation of the liver and pancreas and cancer of these organs,[63] inflammatory bowel disease (IBD) causes colon cancer;[64, 65] *Schistosoma spp.* infection is associated with bladder and colon cancers[66, 67] and chronic viral hepatitis is associated with liver cancer.[68]

The concept of chronic inflammation driving or even initiating cancer development was first proposed by Rudolf Virchow back in 1863.[69] Gilmour and colleagues found that gastric adenocarcinomas are frequently found in areas of chronic inflammation and atrophic gastritis[70] and Correa further suggested that the incidence of atrophic gastritis is closely linked to a greater risk for developing gastric cancer.[71] Interestingly, it has further been proposed that any infectious microorganisms or combinations of microorganisms (bacterial overgrowth) causing chronic inflammation are capable of promoting preneoplastic changes in the stomach,[72] emphasizing an initiating role of chronic inflammation in gastric malignancies.

The presence of *Helicobacter* in the stomach results in a host immune response aiming to swiftly eradicate the invading organisms by phagocytosis or secreted anti-microbial substances.[73] The immune response is characterized by mucosal infiltration of various cells of the innate immune system leading to an acute gastric inflammation (acute gastritis). If the bacteria can be eliminated successfully, acute inflammation is readily resolved and the normal tissue architecture is restored. However, incomplete bacterial eradication leads to a persistent inflammatory process resulting in chronic inflammation.[74] The massive infiltration of T- and B-cells leads to the loss and/or displacement of specialized cell types, to the formation of scar tissue and impairment of organ functions. In addition, inducible nitric oxide synthase (iNOS)- or neutrophil-derived reactive oxygen/nitrogen intermediates (ROI/RNI) and certain cytokines such as interferon (IFN)-γ,[18, 75] tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6[69] produced by the infiltrating immune cells have the potential to support cancer development. A very interesting observation supporting the above stated concept is that factors resulting in intensified immune responses to the *Helicobacter* infection render patients more susceptible to cancer development. Studies on single nucleotide polymorphisms (SNPs) in prominent pro-inflammatory genes leading to higher protein expression show that a combination of SNPs in the IL-1β gene cluster (including IL-1β and IL-1 receptor antagonist (IL-1RN)), TNF-α and IL-10 confer a 27-fold increased risk of gastric cancer in *H. pylori*-infected patients.[7, 76] In combination with high-risk bacterial genotypes, polymorphisms increase the risk up to 87-fold over baseline.[77] On the other hand, the conditional suppression of the NF-κB-mediated pro-inflammatory response to *H.felis* has

been shown to prevent the development of gastric atrophy and dysplasia in mice.[78] Thus, specific suppression of key mediators of the inflammatory process may alleviate and even inhibit *Helicobacter*-induced chronic inflammation and thus prevent gastric carcinogenesis.

1.5 Key Components of the Inflammatory Process Contribute to Gastric Carcinogenesis

Besides the above mentioned host and pathogen-specific pro-carcinogenic factors, COX-2/PGE_2-dependent signalling and Poly(ADP-ribose)polymerase (PARP)-dependent ADP-ribosylation have been shown to critically participate in inflammatory processes and malignant disease rendering them interesting as mediators of gastric carcinogenesis and thus targets for pharmacological intervention. In the following, the two pathways will be described in more detail with focus on their implication during inflammation and *Helicobacter*-associated disease as well as state of the art of pharmacological approaches in the respective fields of investigation.

1.5.1 COX-2 Enzymes at the Interface of Inflammation and Cancer

1.5.1.1 *COX-2/PGE_2, Inflammation and Cancer*

Cyclooxygenase (COX) derived prostanoids are common molecular mediators of inflammation and carcinogenesis. The production of prostaglandins (PGs) is initiated by the liberation of arachidonic acid from membrane phospholipids by phospholipase A2 in response to inflammatory stimuli.[79] PGs are potent bioactive lipid messengers that were originally extracted from semen and prostate by Goldblatt and von Euler in the 1930s, thus explaining their designation. Cyclooxygenase (COX) enzymes are membrane-bound, bifunctional enzymes that catalyze the rate-limiting conversion of AA to Prostaglandin endoperoxidase H_2 (PGH_2), by subsequent cyclooxygenation and peroxidation reactions (Figure 2). PGH_2 is the immediate substrate for a series of cell- and tissue-specific prostaglandin sythases resulting in prostanoids, a collective term for prostaglandins (PGs), leukotriens and thromboxanes (TXAs) (Figure 2).

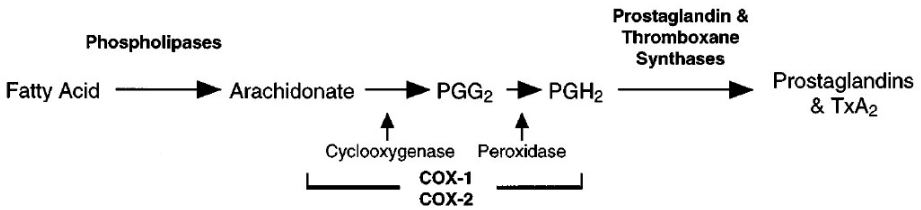

Figure 2 Cyclooxygenase (COX) enzymes catalyze the conversion of arachidonic acid to PGH_2 via a two-step process: in a cyclooxygenation reaction, COX introduces two molecules of oxygen to arachidonate, forming the bicyclic peroxide intermediate prostaglandin G_2 (PGG_2). In the following peroxidation reaction, PGG_2 is reduced to the freely diffusible PGH_2. PGH_2 is a highly unstable endoperoxidase and immediate substrate for a number of cell specific prostaglandin and thromboxane synthases resulting in PGs of the E_2, F_2 and D_2 series and also to PGI_2 (prostacyclin) and thromboxane (TX) A_2 (adapted from Williams *et al.*, 1999).[4]

Three COX isoforms are known to date: COX-1 is present in most cell types and is typically constitutively expressed.[80] An important function in the gastrointestinal (GI) tract includes the regulation of gastrointestinal homeostasis via PGs.[81] COX-1 has recently been recognized to also play a role during carcinogenesis, e.g. of the female reproductive system.[82,83] The second isoform, COX-2, is transiently expressed in most tissues upon

stimulation[84, 85] and primarily regulates inflammatory processes[86] but is constitutively expressed in a variety of epithelial malignancies[87] and has been suggested as prognostic factor for gastric adenocarcinoma.[88] COX-2 is expressed between 2-7 hours before it is degraded by ubiquitination and proteolysis by the 26S proteasome.[89] COX-2-inducing stimuli include LPS,[90-92] IL-1, TNF-α,[93-95] mitogens, IFN-γ[96] and transforming growth factor-alpha (TGF-α)[97] and others. In addition, PGE synthases may itself be directly activated by pro-inflammatory stimuli adding an additional layer of complexity to PG-mediated responses.[98] The third isoform, COX-3 is a splice variant of COX-1 with yet unclear function.[89, 99, 100] PGs play critical roles in numerous biologic processes, including the regulation of immune functions, kidney development, reproductive biology, and gastrointestinal integrity (see Table 2).[101]

In the following, the focus will be on Prostaglandin E_2 (PGE_2), one of the main lipid mediators of COX-2. PGE_2 exerts its function by binding to four different G-protein coupled receptors, the E-Prostanoid receptors 1-4 (EP1-4). In mice, EP receptors are expressed on the plasma membrane of most tissues and EP1 and 4 may also be expressed on nuclear membranes[102]. EP1 couples to Gq/p and regulates intracellular Ca^{2+} concentrations, whereas cAMP is the second messenger of EP2-4.[103] The ubiquitous expression of EP receptors allows PGE_2 to dynamically influence a plethora of physiological and also pathophysiological processes (Table 2).[104] COX/PGE_2-mediated signalling critically participates in cancer development, especially in cancers of the gastro-intestinal tract (Figure 3). This is underlined by the observations that COX-2 is constitutively expressed in a variety of epithelial tumors, such as tumors of the colon, breast, lung, pancreas, esophagus and squamous cell carcinoma[105-108] and correlates with poor prognosis (Juuti, 2006).

Several tumor models have sustained that notion. For one, COX-2 over-expression in rat intestinal epithelial cells (RIE) cells resulted in cellular alterations with potentially tumor-promoting character exemplified by the loss of E-cadherin, increased BCL2 protein levels and reduced transforming growth factor-β (TGF-β) levels leading to increased adhesion and inhibition of apoptosis (Figure 3).[109] Constitutive COX-2 enzymatic activity has been suggested to drive tumorigenic transformation by promoting neovascularisation in murine mammary glands,[110] but also promoting cancer cell metastasis and invasion by regulating matrix metalloproteinase-2 (MMP-2) and CD44,[111] thereby promoting cell invasion.[112]

The role of COX-2 has further been intensively investigated in rodent models of colon cancers. For example, *COX-2* gene ablation in $APC^{\Delta 716}$ mice, a model of human familial adenomatous polyposis (FA), has been shown to lead to dramatically decreased numbers of intestinal polyps[113] and vascular density.[114] Concordant with this observation, other groups have shown that COX-2 derived PGE_2 transactivates PPARδ indirectly via PI3K/Akt, resulting in increased colorectal adenoma growth in APC^{min} mice.[115] Evidence also exists that PGE_2

promotes cell proliferation by two means: either by transactivating Wnt by phosphorylation of GSK-3β[116] or EGF-receptor pathway by the activation of Akt/MAPK.[115, 117]

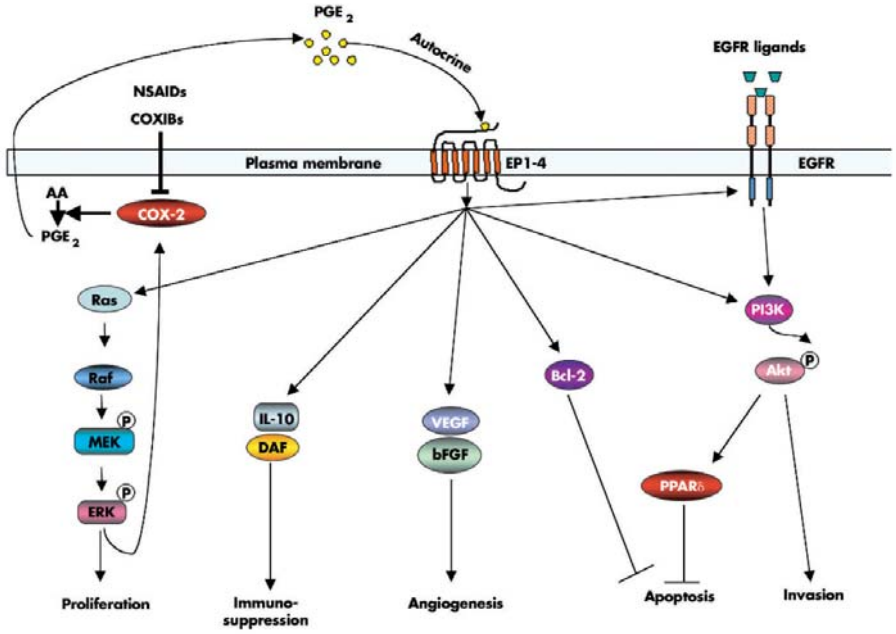

Figure 3 The role of PEG_2 in carcinogenesis. PGE_2 promotes tumor growth by stimulating EP receptor downstream signalling and subsequent enhancement of cellular proliferation, promotion of angiogenesis, inhibition of apoptosis, stimulation of invasion/ motility, and suppression of immune responses.

Abbreviations: NSAIDs, non-steroidal anti-inflammatory drugs; COXIBs, COX-2 selective inhibitors; AA, arachidonic acid; EGFR, epidermal growth factor receptor; IL-10, interleukin 10; DAF, decay accelerating factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; PPARδ, peroxisome proliferator activated receptor δ.

There is also compelling evidence for a role of COX/PGE_2-dependent pathways in *Helicobacter*-associated gastric diseases. *Helicobacter* infection has been shown to lead to elevated levels of COX-2 in infected host cells[118, 119] and in the gastric mucosa of *H. pylori*-infected patients[120] correlating with increased levels of PGE_2 production in these biopsies. Furthermore, Liu and colleagues found that patients were homozygous for the (-1195AA) variant in the *COX-2* regulatory region that affects COX expression levels had a 2-fold higher risk for progressing to gastric cancer than if the variant was heterozygously manifested. The high risk group was further largely represented smokers that were simultaneously infected with *H. pylori*.[121, 122] Moreover, the transgenic expression of COX-2/microsomal PGE synthase 1 (mPGES-1) in mice lead to the spontaneous developed of gastric hyperplasia, tumorous growth and disturbed mucosal differentiation.[123] In addition to the above mentioned pro-tumorigenic properties, the COX-2 byproduct, malondialdehyde, has been shown to directly form DNA adducts resulting in mutations that could initiate carcinogenesis.[82]

Besides inhibition of apoptosis, promotion of cell proliferation and induction of pro-angiogenic pathways, PGE_2 plays a critical role in regulating immune functions[1]: it shapes the T-cell repertoire by regulation of Fas-ligand (FasL) mRNA and protein levels[124] and thereby Fas-mediated apoptosis of $CD4^+$ and $CD8^+$ T-cells.[125] Furthermore, PGE_2 negatively regulates $CD4^+$ and $CD8^+$ T-cell proliferation upon various stimuli[126] and has been shown to bias T helper (Th) cells responses. Experimental data suggest that PGE_2 suppresses Th1 immune responses and drives the overall immune response towards a Th2 polarized response (see Figure 4). Evidence for that comes from studies showing that PGE_2 has no

effect or enhances Th2 cytokines (IL-4, IL-5 and IL-12), but drastically inhibits the production of Th1 cytokines, such as IFN-γ and IL-2, by Th1 cells.[127] Other studies have suggested that PGE_2 also promotes the maturation of regulatory T-cell subsets that suppress effector T-cell activity.[128-130] Moreover, PGE_2 has also been shown to block the initial steps of the adaptive immune response by inhibiting MHC class II expression on professional antigen-presenting cells (APC),[131] and suppressing IL-12 and IL-10 secretion resulting in inhibited dentritic cell (DC) activation in lymph nodes. DC-derived PGE_2 is thought to itself exert immunomodulatory functions.[132]

Besides regulating lymphoid cells of the adaptive immune system, PGE_2 also intervenes with innate immune responses and suppresses IL-12 and TNF-α levels in macrophages.[133, 134]

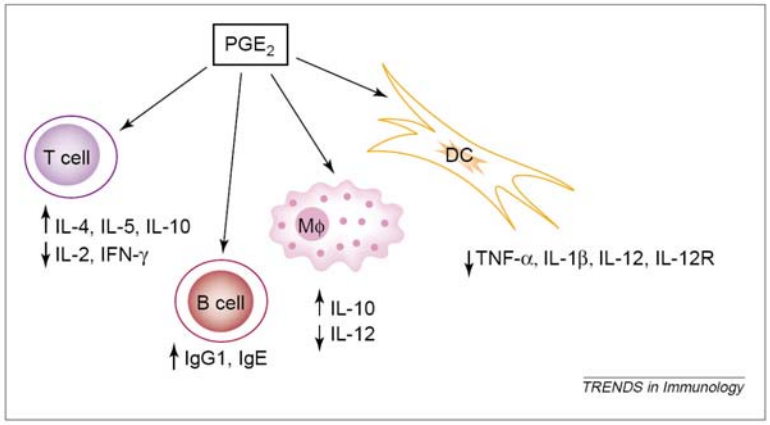

Figure 4. A model for immuno-modulatory functions of PGE_2. PGE_2 enhances the production of type-2 cytokines and antibodies. PGE_2 acts on T cells to enhance their production of in IL-4, IL-5 and IL-10, but inhibits their production of IL-2 and IFN-γ. Acting on B cells, PGE2 stimulates isotype-class switching to induce the production of IgG1 and IgE. PGE_2 acts on antigen presenting cells, such as macrophages (Mφs) and dendritic cells (DCs), to induce the expression of IL-10 and inhibit expression of IL-12, IL-12 receptor (IL-12R), tumor necrosis factor α (TNF-α)and IL-1β. The overall result is an enhancement of T helper 2 (Th2) responses and inhibition of Th1 responses by PGE_2 (adapted from Harris, 2002).[1]

In the context of carcinogenesis, the immune-suppressive potential of COX-2/PGE_2 facilitates evasion of host immune surveillance and anti-tumor responses.[135] Altogether, COX/PGE_2 overexpression in tumors promote carcinogenensis by conferring resistance to apoptosis,[109] increased angiogenesis,[136] decreased host immunity, 2000),[137,138] and enhanced invasion and and metastasis.[111, 112] However, it is still unclear which role COX/PGE_2-dependent signalling plays in premalignant conditions, during the initiation of carcinogenesis and if its immunosuppressive features play a noteworthy role in inflammation-associated cancers. Taken together, the available data strongly support a tumor-favoring role of COX-2/PGE_2 in malignant disease. However, surprisingly little is known about the contribution of COX-2/PGE_2 in precancerous stages and more importantly, in the progression of premalignant to malignant lesions.

1.5.1.2 *COX inhibitors in inflammation and cancer*

An interesting fact proving that COX enzymes indeed play a significant role in tumors is that the regular low-dose intake of non-steroidal anti-inflammatory drugs (NSAIDs) over a period of 10-15 years protects from sporadic colon cancer and other GI cancers,[5, 139, 140] as well as neurodegenerative diseases.[141] Conventional NSAIDs (e.g. Aspirin) are non-selective inhibitors of COX-1 and COX-2 that inhibit the COX enzymatic activity and differentially regulate non-COX target at high doses (e.g. NF-kB and PPARβ/δ) (Figure 5). The protective effect of NSAIDs in cancers has been attributed to the abrogated COX-mediated immune protection of the tumor, as well as to the inhibition of COX-2 is pro-tumorigenic properties and to non-COX specific effects.[142] Already in 1971, Vane and colleagues demonstrated that aspirin and indomethacin-dependent inhibition of inflammation rests primarily on the ability of those drugs to inhibit COX-dependent PG production.[143]

At present, NSAIDs are among the most widely prescribed class of pharmaceutical agents worldwide, having broad clinical utility in treating pain, fever and inflammation.[144] However, traditional NSAIDs (e.g. aspirin, indomethacin, ibuprofen, meclofenamate etc.) have been linked to severe gastrotoxicity due to the fact that COX-1- and COX-2-derived PGs regulate homeostatic functions, such as gastrointestinal cytoprotection and regulation of gastric acidity. To alleviate the negative side effects, COX-2-specific inhibitors (COXibs) have been developed, which bind to the COX-2 active site. Two COXibs, celecoxib (Celebrex) and rofecoxib (Vioxx), have proven beneficial effects in diseases resulting from chronic inflammation, such as osteoarthritis and rheumatoid arthritis, and have also reduced the incidence of gastrointestinal ulcers and erosions seen with standard NSAID therapy.[145-149] In contrast, the influence of NSAIDs on the development of gastric cancers is largely unknown.

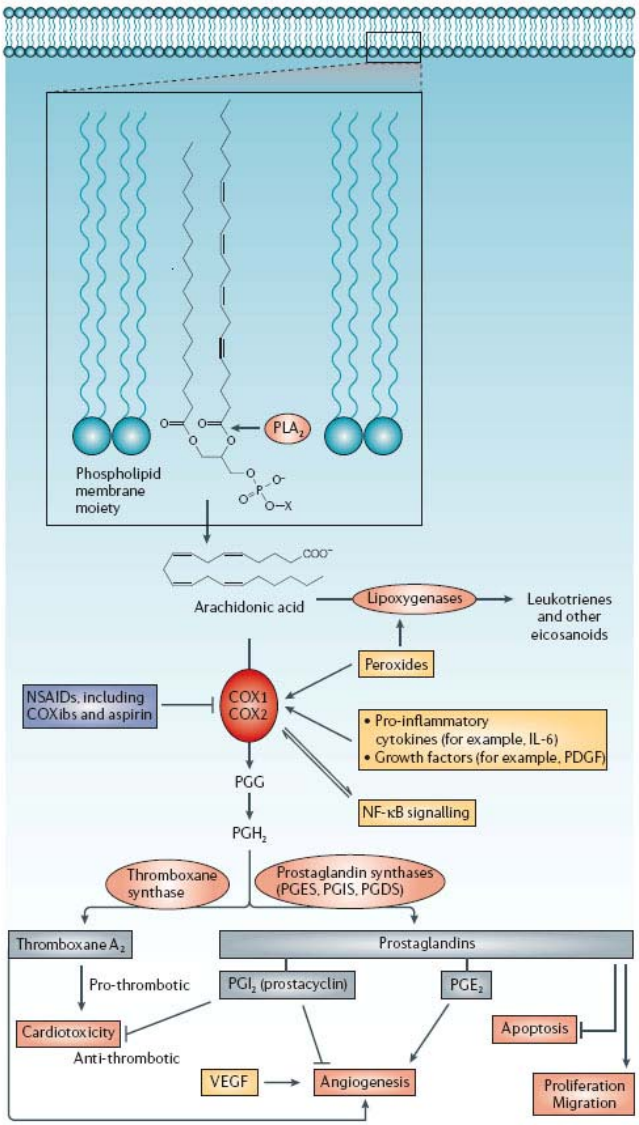

Figure 5 NSAIDs, COX inhibition and prostaglandins. The main targets of COX-2-specific inhibitors (COXibs) and non-steroidal anti-inflammatory drugs (NSAIDs) are COX-1 and COX-2 — enzymes that are central to the conversion of arachidonic acid to prostaglandins. An alternative pathway of arachidonic acid metabolism involves the lipoxygenases. Both the lipoxygenase and COX pathways are regulated by peroxide concentrations, with COX-2 being induced at lower peroxide concentrations than COX-1. Prostaglandins (PGs) influence angiogenesis, apoptosis, cell proliferation and migration. The balance between prothrombotic factors (for example, thromboxane A_2) and anti-thrombotic factors (for example, prostacyclin) might be of particular relevance to cardiovascular toxicity.

Abbreviations: IL-6, interleukin 6; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor;PLA2, phospholipase A2, which cleaves fatty acids, such as arichidonic acid, fromphospholipids; VEGF, vascular endothelial growth factor (adapted from Ulrich *et al*, 2006).[5]

1.5.2 Poly(ADP-ribosyl)ation, Inflammation and Cancer

1.5.2.1 *Poly(ADP-ribosyl)ation and PARPs*

The PARP family of enzymes constitute key players during inflammatory processes and as guardians of genome stability. Poly(ADP-ribose)polymerases (PARPs) mediate the modification of acceptor proteins with Poly-ADP-ribose (PAR),[150] a very rapid and multilateral posttranscriptional modification, also called Poly(ADP-ribosyl)ation or PARylation. The addition of the negatively charged PAR to acceptor proteins is the most drastic post-translational modifications of proteins that is observed in almost all nucleated cells of mammals, plants and lower eukaryotes, but is absent in yeast (for reviews see Diefenbach and Buerkle, 2005).[3] The most important acceptor for PARylation is PARP1 itself[151] at glutamate and lysine residues[152] but a variety of other acceptors have been described such as p53,[153] histones,[154] and DNA-dependent protein kinases.[155] PARP-dependent PARylation modulates protein function by regulating either enzymatic activities or macromolecular interactions with proteins, DNA or RNA or through non-covalent binding.[156]

PARylation is catalyzed by Poly(ADP-ribose) polymerases (PARPs) that are encoded in the human genome by a set of 18 different genes.[157] The prototypic PARP is the constitutively expressed PARP1 (Figure 6), a nuclear chromatin-associated zinc-finger binding protein. PARP2 shares 60% sequence homology with PARP1,[158] possesses less enzymatic activity but is responsible for residual PAR formation in PARP1 deficient cells.[159, 160]

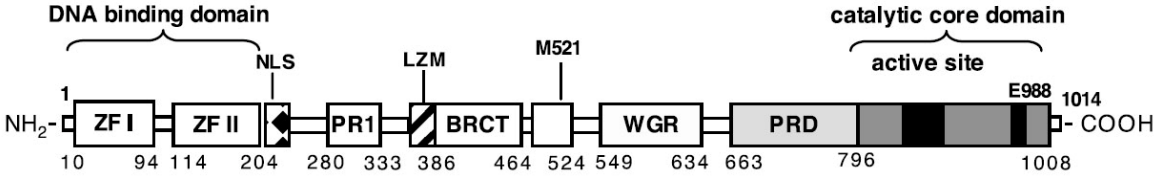

Figure 6 The domain structure of the human PARP1. The most significant domains detected are indicated. The catalytic core domain (also PARP domain) catalyzes Poly(ADP-ribosyl)ation. The PRD domain is called the PARP regulatory domain and may be involved in regulation of the PARP-branching activity. The WGR domain is named after the most conserved central motif (W/G/R) of the domain. This motif is found in a variety of poly(A) polymerases and other proteins of unknown function. The BRCT domain is named after the breast cancer suppressor protein-1 (BRCA1) carboxy-terminal domain and is found within many DNA damage repair and cell cycle checkpoint proteins. The unique diversity of this domain superfamily allows BRCT modules to interact by forming homo- or hetero-BRCT multimers and phosphorylation-dependent BRCT–non-BRCT interactions. The sterile alpha motif (SAM) is a widespread domain in signalling and nuclear proteins and mediates homo- or heterodimerization in many cases. ZF-I and ZF-II, PARP-1-type zinc finger domains (they can act as DNA nick sensors and general DNA-binding domains; NLS, nuclear localization signal (modified from Hassa, 2006).[6]

PARP1 contains three functionally distinct domains: an amino-terminal DNA-binding domain (DBD) containing a zinc-finger-like motif, a central auto-modification domain (AD) containing a BRCA1 carboxy-terminal (BRCT) repeat motif and a carboxylterminal PARP homology domain containing the catalytic domain (active site). The active site mediates NAD^+ hydrolysis, initiation, elongation, branching and termination of PAR synthesis.[161, 162] Interestingly, this domain shows homology with the active site of the bacterial ADP-

ribosylating toxins from *C. diphtheriae, B. pertussis* and *V. cholerae*.[162] Interestingly, the BRCT motif found in the AD domain is also found in other components of the DNA damage checkpoint and repair pathways.

PARPs use the respiratory coenzyme nicotinamide adenine dinucleotide (NAD^+) as a source of ADP-ribose moieties to synthesize protein-bound polymers of variable size (from 2 to more than 200 residues) and structural complexity (linear or branched) resulting in strongly negatively charged PAR (Figure 7).

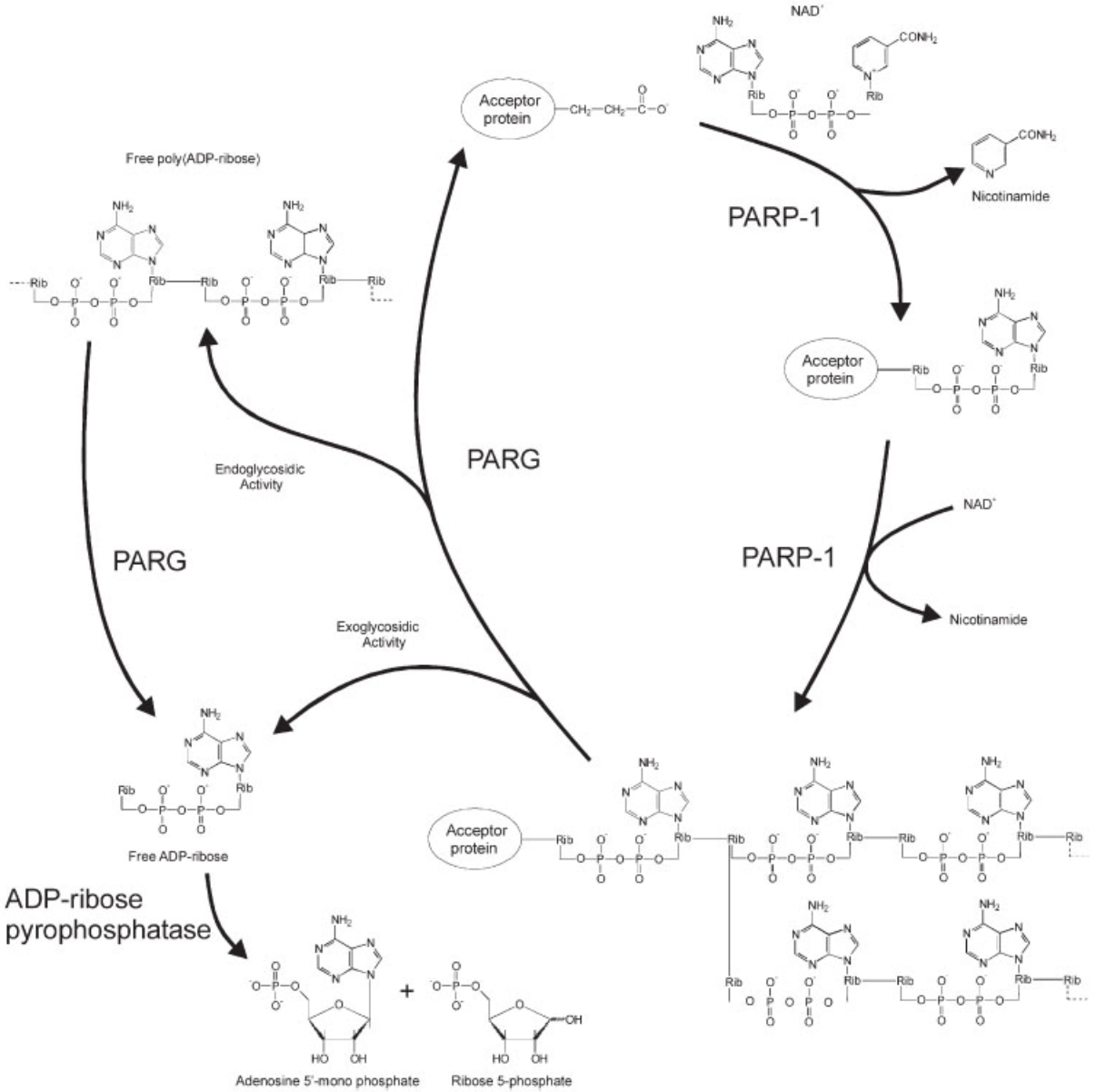

Figure 7 Metabolism of poly(ADP-ribose) (PAR). PARP1 cleaves the glycosidic bond of NAD+ between nicotinamide and ribose followed by the covalent modification of mainly glutamate residues of acceptor proteins with an ADP-ribosyl unit, resulting in the formation of an ester bond between the protein and the ADP-ribose residue. PARP1 also catalyses the elongation and branching reaction, giving rise to polymers with chain lengths of up to 200 ADP-ribosyl units and several branching points. PARG is the only protein known to catalyse hydrolysis of (ADP-ribose) polymers to free ADP-ribose exhibiting endoglycosidic activity along with exoglycosidic activity. PARG was reported to be able to remove even the primary ADP-ribosyl group bound to acceptor proteins, indicating a functional overlap with ADP-ribosyl protein lyase that was shown to remove the primary ADP-ribosyl group from acceptor proteins (from Diefenbach and Buerkle, 2005).[3]

PARP1 catalyzes the formation of ADP-ribose from NAD^+ by cleavage of the glycosidic bond between nicotinamide and ribose.[163] Target proteins are either modified non-covalently or by

covalent modification of glutamate, aspartate and carboxyterminal lysine residues[152, 164] with ADP-ribose subunits. PARP1 further catalyzes elongation and branching using additional ADP-ribose units from NAD^+.[165] These negatively charged polymers can drastically alter the properties of the protein acceptor.

PARP dependent PARylation is induced most efficiently upon DNA damage and is proposed to participate in the DNA damage surveillance network. Together with PARP-2, PARP1 is the only enzyme to be highly stimulated by DNA interruptions, but can also be activated by other DNA structures such as hairpins, cruciforms and supercoiled DNA,[166, 167] suggesting that DNA breaks are not essential for PARP activation. In the absence of DNA single or double-strand breaks, PARylation is a very rare event but reaches an up to 500-fold increase upon DNA damage.[3] PARP1 synthesizes 90% of PAR following DNA breaks.[6, 168]

Furthermore, PARylation has been implicated in various other cellular processes such as transcriptional regulation, telomere cohesion and mitotic spindle formation during cell division, intracellular trafficking and energy metabolism[169] and cell survival and apoptosis. During apoptosis, PARP is involved in the release apoptosis-inducing factor (AIF) from mitochondria which, upon translocation into the nucleus, activates caspase-dependent and independent apoptosis.[170, 171] However, apoptosis occurs also in the absence of PARP.[172]

Intracellular PAR is short-lived and under tight control of the Poly (ADP-ribose) glycohydrolase (PARG)[173] and possibly ADP-ribose hydrolase (ARH3)[174] to avoid energy-depletion by extensive usage of NAD^+ and ATP and to avoid cellular necrosis (PARP suicide model).[175] The two enzymes catalyze the hydrolysis of (ADP-ribose) polymers to free ADP-ribose.[173, 176] PARP1 is cleaved by caspases into a 24kDa N-terminal and 89kDa C-terminal fragment, none of which is able to respond to DNA nicks any longer, thereby enabling NAD^+ pools to be restored.[173, 177]

However, PARP1 does not play essential roles in any of the processes stated above since PARP1 deficient mice are healthy, fertile and do not have any pre-disposition to develop spontaneous tumors although they show hypersensitivity to DNA-damaging agents.[178]

1.5.2.2 *PARP, PAR and their role in DNA damage responses*

As already mentioned, PARPs are activated upon DNA damage and participate in a signalling network developed by the eukaryotic cell to cope with numerous environmental and endogenous genotoxic agents and to preserve genome integrity. PARylation of PARP1/2 following genotoxic insult such as reactive oxygen species (H_2O_2), irradiation (IR)-induced DNA strand breaks, alkylating agents, or intermediates of repair processes serves as the signal to initiate DNA repair or cell death:[161] DNA relaxation by PARylation of histones, negatively charged PAR changes binding affinities of proteins (to DNA, other proteins etc.),

recruitment of repair enzymes to sites of damage and others.[179] Upon damage, PARP1 enzymes play experimentally proven roles in DNA damage sensing, signalling and repair.[180-184] PARP1 interacts with a number of proteins involved in DNA repair: XRCC1,[185, 186] DNA liagase III , DNA polymerase β,[187] Ku 70/80[188] and PCNA.[189] Many of those proteins have been shown to bind PAR *in vitro*[187, 190] and may even be targets of covalent PAR interactions.[191] The important, but not essential, role of PARP-1 in DNA damage signalling is supported by the fact that PARP1 null mice are hypersensitive to ionizing radiation and N-Methyl-N-nitrosourea (MNU) treatment.[192] However, this finding is challenged by the fact that PARP1 deficient mice are viable, fertile, have a normal lifespan and do not develop spontaneous tumors.[178, 193]

1.5.2.3 *PARPs, PAR and their role in inflammation*

More important in the context of inflammation-associated carcinogenesis is the notion that the activation of PARPs plays an important role in various models of chronic inflammation. PARP1 is an important co-activator of nuclear factor kappaB (NF-kB) and thereby indirectly impacts on the transcription of pro-inflammatory genes, such as cytokines, chemokines, adhesion molecules and inflammatory mediators.[194] Equally, *PARP1* deficient mice are astoundingly resistant to experimentally induced inflammation and injury: *PARP1* deficient mice are resistant to endotoxic shock as a consequence of defective NF-κB activation and consequently TNF-α and IFN-γ secretion;[195] PARP1 deficiency further protects mice from LPS-induced septic shock,[196] streptozotocin-induced diabetes mellitus,[197] allergen-induced airway inflammation[198] and collagen antibody-induced arthritis.[199]

In addition to NF-κB-dependent processes, PARP1 regulates the expression of a set of genes important for cell adaptation to hostile environments, such as inflammation (e.g. in neutrophils)[200] and hypoxic conditions, by forming a functional complex with hypoxia inducible factor-1 (HIF-1) and promotes the respective gene expression.[201]

PARP1 was recently further assigned a role in T-cell activation through regulation of the nuclear factor of activated T cells (NFAT) family of transcription factors,[202, 203] however, it remains unclear if this effect depends on PARP enzymatic activity.

So far, however, only little is known about the relation of PARP and PARylation in gastric diseases. Recently, Zhang *et al*. showed that the PARP1 762Ala/Ala polymorphism, previously reported to correlate with increased susceptibility to prostate cancer (Lockett, 2004), was slightly over-represented in patients with gastric cancer (16.9%) compared to disease-free individuals (10.3%).[204] Another recently published study reports PARP1 activation by *Helicobacter* infection in cell lines *in vitro.*[205] So far, however, the role of PARPs and PARylation has not been addressed in detail in mouse models of gastric diseases and cancer progression.

1.5.2.4 *Inhibitors of Poly(ADP-ribosyl)ation, Inflammation and Cancer*

The importance of PARP in different physiological processes has further been confirmed by pharmacological inhibition of PARP enzyme activity. As already mentioned, PARylation is catalyzed by PARP enzymes, utilizing NAD^+ as substrate. NAD^+ binds into a cleft located in the PARP1 catalytic fragment (CF) and catalyzes the PARylation reaction resulting in nicotinamide as byproduct.

Nicotinamide is a well-known inhibitor of PARP and physiologically acts as endogenous feedback inhibitor.[206] The first non-selective inhibitor of PARP activity, 3-aminobenzamide, has been described 30 years ago.[207] A variety of compounds mimicking the nicotinamide-moiety of NAD^+ have been designed ever since to inhibit PARP enzymatic activity and thus poly(ADP-ribosyl)ation as for example PJ34 (Figure 8). Though being an endogenous coenzyme, it is also a substrate for other NAD-metabolizing enzymes and may encompass off-target effects. Various PARP inhibitors have shown disease-relieving effects in a variety of chronic inflammation-associated diseases: it abolished systemic pro-inflammatory responses to thoracic aortic ischemia and reperfusion,[208] attenuated inflammation in a model of chronic colitis (Crohn's disease)[209] and in allergen-induced airway inflammation models,[198] and protected mice from endotoxic shock[210] and experimentally-induced diabetic neuropathy.[211]

The water-soluble phenanthridinone derivative PJ34 (Figure 8)[2] has been shown to block the increase of IL-6, intercellular adhesion molecule-1 (ICAM-1), E-selectin and TNF-α, IL1-β mRNA and/or protein levels in different models, accompanied with reduced infiltration of immune cells into the respective tissues.[212-215] These observations conform to the fact that PARP1 deficient cells show suppressed transcript levels of those genes.[195, 212-217]

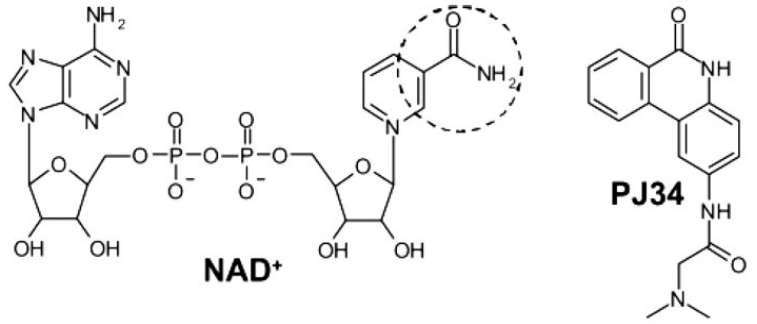

Figure 8 Structures of NAD^+ and the competitive PARP-1 inhibitor PJ34. PJ34 is a water-soluble phenanthridinone derivate. The aromatic ring-linked carboxamide group (circled) or carbamoyl group built in a polyaromatic heterocyclic skeleton is shared by the natural PARP1 substrate NAD^+ and the competitive PARP-1 inhibitors nicotinamide and PJ34 (modified from Alano, 2006).[2]

Cancer therapies, such as radiotherapy/cytotoxic chemotherapeutics, aim to massively and specifically damage the DNA of cancer cells and thereby eliminate them by programmed cell death.[218] However, normal tissue may also be affected by the treatment leading to the development of new cancers in regions surrounding the tumorous tissue. To address this problem, major efforts have been made to develop cancer type-specific

therapies by targeting tumor cells carrying tumor-specific features such higher proliferation rate, the BCR/ABL fusion protein[219] or HER-2[220] over expression. Defects in DNA repair pathways lead to multiple hereditary cancer susceptibility syndromes (for a review see Spry *et al.,* 2007)[221] and the efficacy of DNA damaging agents depends on the DNA repair ability of the targeted cell. In the light of that, a variety of DNA repair-inhibiting agents have been proven to potentiate the efficacy of cancer therapeutics and have been tested in clinical trials: PARP inhibitors,[222] O-6-methylguanine methyltransferases (MGMT) and checkpoint kinase (CHK) 1 and 2 inhibitors.[223] PARP inhibitors in combination with radiotherapy have shown convincing effects in treating tumors defective in homologous recombination (HR) (e.g. BRCA1- and BRCA-2 deficient tumors).[224-226] Furthermore, cells that are defective in recombination-related proteins other than BRCA1 or BRCA2, such as RAD51, RAD54, XRCC2,XRCC3, replication protein A1 (RPA1), ATM, ATR, CHK1, CHK2, NBS1 and components of the Fanconi anaemia repair pathway, also show increased sensitivity to PARP inhibition.[224, 227, 228] These observations suggest that PARP inhibitors might also be suitable in treating several types of tumors with defects in HR.

1.6 The Dynamics of Gastric Carcinogenesis: Tumor-promoters against Guardians of Genome Integrity

Genetic insults by carcinogens leading to alterations in tumor suppressor genes and oncogenes are the most important driving forces of carcinogenesis. Mammalian cells have evolved elaborate systems to avoid the promotion of erroneous genetic information and are able to deal with severe forms of DNA damage. This surveillance system is composed of a DNA damage recognition/DNA damage signalling and a DNA damage response network. In the next paragraph, some of the known gastric tumor promoting factors will be discussed followed by an overview on the most detrimental DNA lesions that occur in a cell, the DNA double strand breaks (DSB), as well as the known DSB response and repair mechanisms.

1.6.1 Promoters of Gastric Carcinogenesis

Activation by tumor-promoters or oncogenes and inactivation of tumor suppressor genes either by mutations, promoter hypermethylation or overexpression result in carcinogenesis.

In the case of gastric cancer, there is no apparent 'orderly' sequence of genetic mutations that accumulate in colorectal carcinogenesis, no mutational events are consistently associated with intermediate steps in the progression to intestinal-type gastric adenocarcinoma. Frequently, however, mutations in the *TP53* gene correlate with relatively early stages of infection. Mutations in *RAS* and 'deleted in colorectal cancer' (DCC) occur in metaplastic, dysplastic and cancerous lesions.[7]

TP53 mutations have been detected in over 60% of advanced gastric cancers, as well as in intestinal metaplasia (38%), gastric dysplasia (60%), and early gastric cancer; and these mutations are associated with a generally worse prognosis.[229, 230] Furthermore, epigenetic silencing by hypermethylation of CpG islands in promoter regions of genes involved in maintaining genetic stability and minimizing mutagenesis has been reported for the mismatch repair protein MLH-1,[231, 232] E-cadherin[233] and p16 ^{INK4A} tumor suppressor gene .[234] Another feature that may facilitate acquisition of mutationsincludes the observation that mismatch repair (MMR) proteins MLH1, PMS1, PMS2, MSH2 and MSH6 are down-regulated in *Helicobacter*-infected individuals, an effect that is reversed by eradication therapy.[235, 236] However, it is unclear if this effect is not solely due to cell cycle arrest and subsequent inhibition of protein synthesis. A possible mechanism of the potential *Helicobacter*-mediated mutagenic role is that *Helicobacter* infection of AGS cells up-regulates activation-induced cytidine deaminase (AID) expression[237] leading to GC to TA transversions. In addition, AID transgenic mice indeed showed increased occurrence of preneoplastic lesion and AID up-regulation correlated with higher *p53* mutation rates in cell lines.[237]

Several studies suggest an association between oxidative DNA damage and *Helicobacter* infection and have observed activated antioxidant defences in gastric cells following infection.[238-240] Cellular oxidants comprise reactive oxygen (ROS) and nitrogen species (RNS). Endogenous sources of ROS include oxidative phosphorylation in mitochondria, cytochrome P450 activity, peroxisomes and the antimicrobial oxidative burst of phagocytic cells (e.g. macrophages, neutrophils and eosinophils).[241] Cells, however, have evolved enzymes such as catalase and superoxide dismutase which can detoxify reactive oxygen species by converting them into water and oxygen. The situation in which ROS exceed cellular antioxidant defences is termed oxidative stress and leads to several types of DNA modifications: DNA-protein crosslinks, abasic sites, purine-reactive aldehyde adducts and oxidized DNA bases.[242] One of the most abundant lesions is 8-oxo-7,8 dihydroguanine (8-oxoG), a commonly used biomarker for DNA oxidation. Because 8-oxoG can pair with cytosine and adenine bases during DNA synthesis, it causes G:C to T:A transversions[243] when present in the template DNA and it induces A:T to C:G transversions when 8-oxoG in the nucleotide pool is incorporated into DNA.[244] Interestingly, elevated levels of 8-oxoG levels have been reported in gastric cancer, intestinal metaplasia and atrophy.[238, 245] Izotti *et al.* also provide evidence that homozygous Ser326Cys polymorphism in the 8-oxoguanosine-glycosylase1 (*OGG1*) gene, encoding the enzyme removing 8-oxoG from DNA, leads to a significant increase in the occurrence of oxidative DNA damage in *H. pylori* infected patients.[238, 245] All these factors together show a correlation of *Helicobacter* infection with possible pro-tumorigenic potential. However, the ultimate proof and actual contribution to carcinogenesis remains unclear and a possible direct genotoxic role of *Helicobacter* products remains to be elucidated.

1.6.1.1 *Double Strand Breaks (DSB)*

The most detrimental of all lesions, however, are DNA double strand breaks (DSB) that can promote genetic instability. Complex signal transduction, cell-cycle checkpoint and repair pathways respond to and repair lesions that are generated by exogenous or endogenous DNA-damaging agents. IR for example induces DSB of high complexity, involving multiple clustered sites of damage (e.g. damaged bases, sugar phoshphates and/or single strand breaks (SSB)) that are introduced directly by breakage of the sugar backbone and indirectly by generating ROS.[242] Endogenously generated ROS are less likely to induce severe DNA damage, but DSB may arise from SSB in close proximity to one another. In addition, DSB can also form in a programmed manner during genetic diversification processes: V(D)J (variable, diversity, joining) recombination (=antigen-independent diversification) ensures highly diverse B, and T-cell receptor repertoires; class-switch recombination (CSR) (=antigen-dependent diversification) generates different

immunoglobulin (Ig) isotypes of the G, A or E class[246] and during meiosis to form genetically variable gametes.[247]

Little is known about the context of bacterial infections and DSB induction. Interesting to note is a recent study showing that commensal and extraintestinal *E.coli* induces DSB beaks apparent as γH2AX foci. DSB occurrence depended on a genomic island coding for non-ribosomal peptide and polyketide synthases. In addition to γH2AX foci, polyketide synthase positive bacteria induced cell cycle arrest in G2/M phase.[248] Furthermore, the group showed that *E.coli* infection also leads to γH2AX foci in colons of BALB/c mice *in vivo*, causing aneupleudy and tetrapleudy that resulted in increased mutation frequencies in various genes.[249] Despite its widely accepted role in gastric carcinogenensis, the existence of DNA damaging factors has never been elucidated in *Helicobacter* spp..

1.6.2 The Surveillance Network

1.6.2.1 *The Guardians I: DSB - Induced Signal Transduction Pathways*

Several redundant pathways alert the cell to the presence of DNA damage and subsequently coordinate an appropriate response.[250] Components of the DSB-induced signal transduction pathways include the DNA-dependent protein kinase (DNA-PK), the phosphatidylinositol 3-kinase related kinases (PIKKs) – ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). The main DSB repair pathways include DNA non-homologous end joining (NHEJ) and homologous recombination (HR). The primary sensor of DSB is the MRE11-RAD50-NBS1 complex (MRN) which recruits ATM to the DSB (Falck, 2005). In another early step, H2A histone family member X (H2AX) is phosphorylated (γH2AX) redundantly by ATM or DNA-PK.[251] H2AX phosphorylation happens over megabase-pair regions being apparent as microscopically detectable foci.[252] γH2AX mediates the recruitment of and retains mediator proteins such as p53 binding protein-1 (53BP1), mediator of DNA damage checkpoint 1(MDC1), breast cancer 1 (BRCA1) and the MRN complex at the site of DSB.[253] ATM regulates cell cycle arrest at different checkpoints after IR: G1-S arrest allows cells to repair DNA before replication and is dependent on P53 and checkpoint-2 (CHK2) proteins and requires activation of p21, the inhibitor of cyclin-dependent kinase (CDK) and intra-S-phase and G2 arrest via FancD2, p53 and E2F1.[254] It is interesting to note, that ATM and ATM-dependent pathways (CHK2, H2AX and p53) are activated already in precursor lesions of some cancers, such as the urinary bladder, breast, lung and colon, and are believed to delay and even prevent cancer formation. In line with this hypothesis, is the observation that loss of heterozygosity (LOH) and mutations in the very same genes and proteins predispose to cancer formation and are present in later stage tumors.[255] In accordance, ATM kinase targets p53, CHK1, CHK2, H2AX, NBS1, BRC1 and

53BP1 qualify as tumor promotors while targets of ATM itself or its down-stream kinases CHK1 and CHK2, such as Mdm2 and Cdc25A are known proto-oncogenes.[256-258] This suggests that DNA damage response fulfil an anti-cancer function in early tumorigenesis.

The second DSB-induced pathway mediated by ATR is activated by single stranded regions of DNA that arise as a result of replication-fork stalling, or during repair of bulky lesions.[259]

1.3.3 *The Guardians II: DSB Repair Pathways*

Breaks in both DNA strands potentially lead to loss of sequence information that cannot be recovered from the same DNA molecule. To avoid DSB-induced loss of genetic information and subsequent disruption of vital cellular processes (e.g. replication, transcription), cells possess robust mechanisms to repair DSBs (Figure 9)[8]. ATM and ATR signalling induce cell-cycle checkpoints to allow repair process to take place.

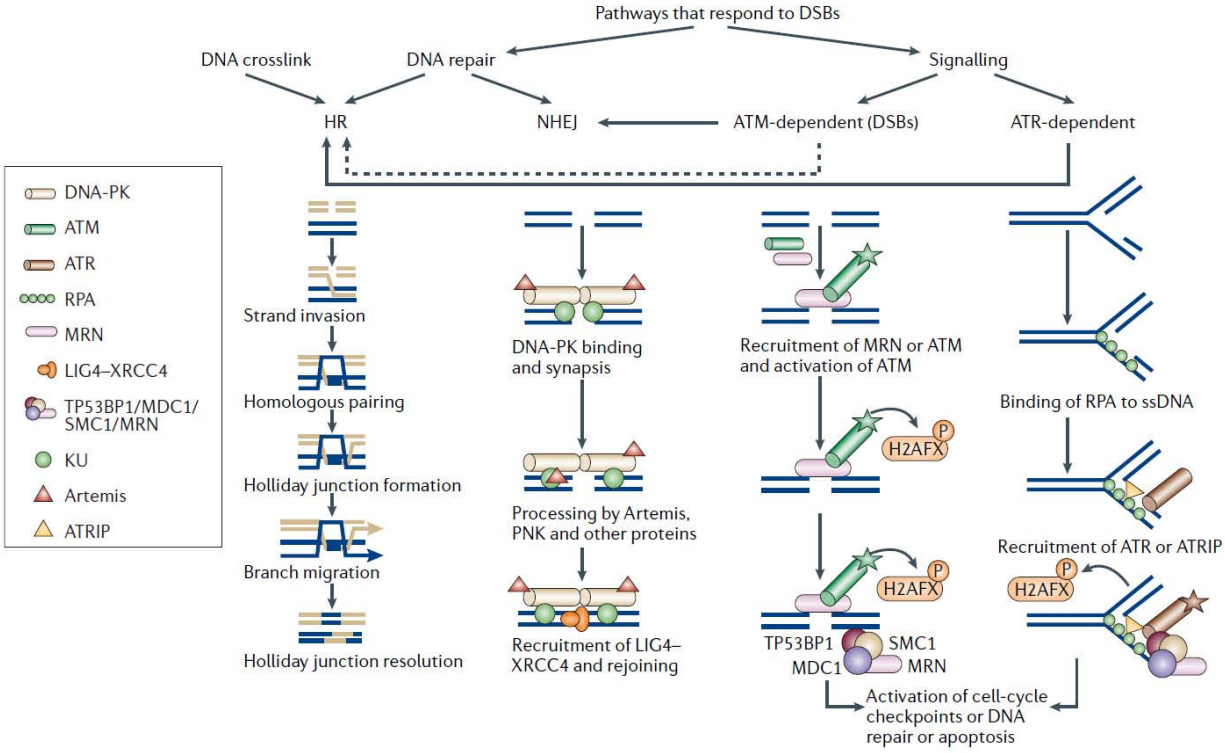

Figure 9. Pathways that respond to double strand breaks. Homologous recombination (HR) takes place in late S–G2 phase and involves the generation of a single-stranded region of DNA, followed by strand invasion, formation of a Holliday junction, DNA synthesis using the intact strand as a template, branch migration and resolution. Non-homologous end-joining (NHEJ) takes place throughout the cell cycle and involves binding of the KU heterodimer to double-strandedDNA ends, recruitment of DNA-PKcs, processing of ends and recruitment of the DNA ligase IV (LIG4)–XRCC4complex, which brings about ligation. The ataxia telangiectasia mutated (ATM) protein affects NHEJ as it is required for Artemis-dependent end processing. ATM signalling is the main signal-transduction process that responds to a DSB. Ataxia telangiectasia and RAD3-related (ATR) signalling is activated later after irradiation, probably when radiation-inducedlesions block replication. Both pathways lead to cell-cycle-checkpoint activation, which allows more time for repair or permanently prevents the proliferation of damaged cells. An important step in ATM- and ATR-dependent signalling is phosphorylation of H2A histone family, member X (H2AFX) and recruitment of the mediator proteins, mediator of DNA damage checkpoint 1 (MDC1), tumor protein 53 binding protein 1 (TP53BP1) and the MRN (MRE11–RAD50–NBS1)complex. Additional proteins, such as structural maintenance of chromosomes 1 (SMC1), are also recruited to the site of damage. Other proteins that are recruited to the foci, such as replication protein A (RPA) and Bloom syndrome (BLM),have not been shown for clarity. ATR signalling is activated by single-stranded regions of DNA that arise, for example, at stalled replication forks. RPA coats single-stranded DNA and recruits ATR and its partner ATRIP (ATR-interacting protein).H2AFX phosphorylation, recruitment of mediator proteins and downstream phosphorylation occur in a process that is similar to the ATM-dependent pathway, although with some distinctions (for example, CHEK1 and CHEK2 (checkpoint 1and 2) might be differently phosphorylated by ATM versus ATR). HR probably depends on ATR, either to stabilize stalled replication forks and/or to activate the process of HR. DNA crosslinks are repaired through a process that probably generates a DSB as an intermediate (adapted from Driscoll, 2008).[8]

The type of repair depends on the manner by which DSBs were created and the phase of the cell cycle. Two-ended DSB, induced for example by IR, can be re-joined through nonhomologous DNA end-joining (NHEJ) throughout the cell cycle (Figure 9)[8]: DNA ends are bound by Ku70/80 dimers which attract DNA-PK to ends and promotes their juxtaposition. If no end-processing is required, the NHEJ core complex consisting of XRCC4, DNA ligase IV and XLF can complete rejoining. End-processing is accomplished by Artemis (ATM-dependent), a nuclease and/or DNA polymerases TdT, pol lambda, and pol μ and also Ku itself.[260]

Homologous recombination (HR) restores the continuity of broken DNA using a homologous, intact template, the sister chromatid, to replace the lost information. HR takes place in late S-G2 phase and involves the generation of single-stranded DNA overhang followed by strand invasion and formation of a D-loop and Holliday junction, DNA synthesis using the intact sister strand as a template, branch migration and resolution (Figure 9). The core protein complex of the HR machinery is encoded by the RAD52 group of genes (for review: Modesti, 2001).[261]

2. Aim of the Study

Gastric adenocarcinoma belongs to the most common causes of cancer-related deaths world-wide and develops as a consequence of chronic inflammation of the stomach lining that is caused by persistent infection with the bacterium *Helicobacter pylori*. In humans as well as in mice, gastric carcinogenesis progresses through a sequence of preneoplastic lesions which manifest histologically as atrophic gastritis, epithelial hyperplasia, intestinal metaplasia and dysplasia. Bacterial eradication shows beneficial effects in people with certain gastric precancerous lesions. However, to date there is no alternative therapy available for patients refractory to eradication, nor is there a sustainable cure for intestinal metaplastic lesions or for the prevention of malignant progression. We and others have shown previously that *Helicobacter*-induced pathogenic $CD4^+$ T-effector cells and their secreted cytokine products are the central mediators of gastric preneoplasia.

The aim of this study was to identify therapeutic molecular targets that regulate the pathogenic T-cell response and to impede the progression from *Helicobacter*-induced precancerous to malignant lesions by targeting pathogenic T-cells. To address this question, we utilized a C57BL/6 mouse model of *Helicobacter felis* infection. Infected mice develop severe gastritis and histologically evident precancerous lesions, such as epithelial hyperplasia and metaplasia, as early as three months post infection. The mice were treated with one of two pharmacological compounds either starting at the time of infection or once cancer precursor lesions were present to assess possible therapeutic effects. The effect of the treatments was quantified by histopathological scoring of gastric cancer precursor lesions and by examining the effect on T-cell proliferation, cytokine secretion and migration *ex vivo*.

Another aim of this study focused on investigating *Helicobacter pylori*'s intrinsic genotoxic potential and its direct contribution to carcinogenesis. To this end, we infected primary epithelial cells and various cell lines with *Helicobacter pylori.* We assessed the integrity of the host cell DNA by pulse field gel electrophoresis (PFGE) and also examined the induction of DNA damage response pathways upon infection.

3. Results

3.1 Published Research Articles in Peer Review Journals

3.1.1 Prostaglandin E_2 Prevents *Helicobacter*-induced Gastric Preneoplasia and Facilitates Persistent Infection in a Mouse Model

Authors: Isabella M. Toller, Iris Hitzler, Ayca Sayi and Anne Mueller

Journal: Gastroenterology 2010; 138:1455-1467
doi:10.1053/j.gastro.2009.12.006

Contribution: Planning, performing and analysis of experiment, except Figure 6A-5 with IH; figure assembly, assistance in manuscript writing, revision of the manuscript (with IH)

Summary: COX-2/PGE_2 dependent signalling plays a crucial role during the inflammatory response raised against invading organisms but is also critically implemented in tumor development and maintenance. The proposed immunosuppressive effects of PGE_2 on $CD4^+$ T cells in the tumor environment that ensuring immune evasion prompted us to examine the role of COX-2/PGE_2 in our model of *Helicobacter*-induced, T-cell-driven gastric preneoplasia in a C57BL/6 mouse model. We found that inhibition of COX-2 enzymatic activity strikingly accelerated development of gastritis and premalignant gastric lesions and that, conversely, systemic administration of synthetic PGE_2 prevented premalignant changes and vaccine-induced protection during *Helicobacter* challenge infection. The protective effects of PGE_2 in our model could be attributed to its immunosuppressive effects on $CD4^+CD25^+$ T cells, which failed to migrate, to proliferate, and to secrete IFN-γ when exposed to PGE_2 *in vitro* or *in vivo*. Taken together, these data suggest that COX-2/PGE_2-dependent pathways are critical in preventing excessive gastric immunopathology by regulating the activity of “pathogenic” T cells.

Prostaglandin E2 Prevents *Helicobacter*-Induced Gastric Preneoplasia and Facilitates Persistent Infection in a Mouse Model

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BACKGROUND & AIMS: Persistent infection with the human pathogen *Helicobacter pylori* increases the risk of gastric cancer. In this study, we investigated the role of cyclooxygenase-2 (COX-2) and its main product, prostaglandin E_2 (PGE_2), in the development of *Helicobacter*-induced gastritis and gastric cancer precursor lesions. **METHODS:** We utilized mouse models of *Helicobacter*-induced gastric preneoplasia and vaccine-induced protection to study the effects of COX-2 inhibition and PGE_2 treatment on the induction of *Helicobacter*-specific immune responses and gastric premalignant immunopathology. **RESULTS:** COX-2 and PGE_2 are up-regulated upon *Helicobacter* infection in cultured epithelial cells and in the gastric mucosa of infected mice. Inhibition of COX-2 activity with celecoxib significantly accelerated early preneoplasia; conversely, systemic administration of synthetic PGE_2 prevented development of premalignant pathology and completely reversed preexisting lesions by suppressing interferon-γ production in the infected stomachs. The protective effect of PGE_2 was accompanied by increased *Helicobacter* colonization in all models. All in vivo effects were attributed to immunosuppressive effects of PGE_2 on $CD4^+$ T-helper 1 cells, which fail to migrate, proliferate, and secrete cytokines when exposed to PGE_2 in vitro and in vivo. T-cell inhibition was found to be due to silencing of interleukin-2 gene transcription, and could be overcome by supplementation with recombinant interleukin-2 in vitro and in vivo. **CONCLUSIONS: COX-2–dependent production of PGE_2 has an important immunomodulatory role during *Helicobacter* infection, preventing excessive local immune responses and the associated immunopathology by inhibiting the effector functions of pathogenic T-helper 1 cells.**

Keywords: Helicobacter pylori; Gastric Cancer; Cyclooxygenase; Prostaglandin E_2.

Persistent gastric colonization with the human pathogen *Helicobacter pylori* causes chronic active gastritis in infected individuals and has been linked epidemiologically to gastric and duodenal ulcers, gastric mucosa-associated lymphoid tissue lymphoma, and gastric cancer.[1–3] The chronic gastric inflammation induced by *H pylori* infection constitutes a prerequisite for the formation of preneoplastic and malignant lesions, both in humans and in rodent models of *Helicobacter*-induced disease.[4,5] The transformation from normal mucosa to gastric cancer occurs via a sequence of precursor lesions starting with atrophic gastritis, intestinal metaplasia, and dysplasia.[6] Using mouse models of experimental *Helicobacter* infection and vaccine-induced protection, we have shown recently that stomach-infiltrating $CD4^+CD25^-$ interferon-γ–secreting T cells play a dual role in the *Helicobacter*–host interaction. On the one hand, these cells are crucial in controlling the infection; on the other hand, they promote development of *Helicobacter*-induced gastric cancer precursor lesions, possibly through a direct effect of IFN-γ on gastric epithelial cells.[7]

The 2 processes of inflammation and carcinogenesis share a common molecular mediator, the cyclooxygenase (COX) enzymes. The COX-1 isoform is constitutively expressed in most cell types, whereas COX-2 is induced in response to proinflammatory stimuli, such as lipopolysaccharide, interleukin-1, and tumor necrosis factor–α.[8] The most important lipid product of COX-2, prostaglandin E_2 (PGE_2), exerts its effects by binding to the E-prostanoid receptors 1–4, which are expressed ubiquitously in most murine tissues.[9]

Several lines of evidence indicate that COX-2–dependent pathways play a role in carcinogenesis, especially in the gastrointestinal tract. Many solid tumors overexpress COX-2/PGE_2, and polymorphisms in the *COX-2* regulatory region determine the carrier's risk of developing several epithelial cancers.[10,11] The long-term low-level intake of nonsteroidal anti-inflammatory drugs inhibiting COX activity reduces the risk for colorectal cancer,[12,13] as well as gastric cancer.[14] Tumor-derived PGE_2 is believed to promote cancer progression by stimulating cell motility/invasion and tumor-associated angiogenesis and by preventing tumor cell apoptosis (reviewed in Cha and DuBois[15] and Wang and DuBois[16]). Several pathways and signaling networks are activated or enhanced by PGE_2;

Abbreviations used in this paper: CFSE, carboxyfluorescein succinimidyl ester; COX, cyclooxygenase; IFN, interferon; IL, interleukin; MLN, mesenteric lymph node; PGE_2, prostaglandin E2; IP, intraperitoneal; PAI, pathogenicity island; Th1, T-helper 1; Treg, regulatory T cell.

0016-5085/10/$36.00
doi:10.1053/j.gastro.2009.12.006

most notably, PGE_2 potentiates Wnt and peroxisome proliferator–activated receptor-δ signaling to induce target genes involved in replication and survival and triggers cell migration by activation of the epidermal growth factor receptor pathway.[15] In addition to its direct effects on cancer cell biology, PGE_2 is known to have strong immunosuppressive effects, giving rise to the hypothesis that PGE_2 production may allow tumors to evade immune surveillance.[17]

The reported immunosuppressive effects of PGE_2 on $CD4^+$ T cells, in particular T-helper 1 (Th1)-polarized effector T cells (reviewed in Harris and Phipps[18]), prompted us to examine the role of COX-2/PGE_2 in our model of *Helicobacter*-induced, T-cell–driven gastric preneoplasia. We found that inhibition of COX-2 enzymatic activity strikingly accelerated development of gastritis and premalignant gastric lesions and that, conversely, systemic administration of synthetic PGE_2 prevented premalignant changes. The protective effects of PGE_2 in our model could be attributed to its immunosuppressive effects on $CD4^+CD25^-$ T cells, which failed to migrate, to proliferate, and to secrete IFN-γ when exposed to PGE_2 in vitro or in vivo. Taken together, these data suggest that COX-2/PGE_2-dependent pathways are critical in preventing excessive gastric immunopathology by regulating the activity of "pathogenic" T cells.

Materials and Methods

Mice, Cell Culture, Bacterial Infections, and Vaccination

Male C57BL/6, interleukin (IL)-$10^{-/-}$BL/6, and Ly5.1BL/6 mice (Charles River Laboratories, Sulzfeld, Germany) were bred at a *Helicobacter*-free and specific pathogen-free facility and infected at 5 to 6 weeks of age. All animal experiments were approved by the cantonal veterinary office. *Helicobacter* strains used for animal experimentation were *H felis* CS1 (ATCC 49179)[19] and *H pylori* strain SS1.[20] Mouse infections were performed orally with $\sim 10^8$ bacteria. Primary gastric epithelial cells were isolated as described previously[21]; the immortalized murine gastric epithelial cell line used in this study was also described previously.[7] Both cell types were grown in Dulbecco's modified Eagle medium/Ham's F12 medium (1:1; Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum and infected for 12 hours with *H pylori* strains G27 or an isogenic mutant lacking the Cag pathogenicity island (PAI), G27ΔPAI.[22] Vaccination of mice was performed as stated in the Supplementary Materials and Methods.

Pharmacological Treatments

For inhibition of COX-2, mice were fed a diet supplemented with 1500 ppm celecoxib (for details see Supplementary Materials and Methods; Research Diets, New Brunswick, NJ) or an identical control diet without the active compound. For PGE_2 treatment in vivo, 10 μg 16,16-dimethyl PGE_2 and 17-phenyl trinor PGE_2 were diluted in 100 μL phosphate-buffered saline (details in Supplementary Materials and Methods; Cayman Chemicals, Ann Arbor, MI) and injected twice intraperitoneally (IP) and once combined IP/orogastrically per week. IL-2 (BD Pharmingen, San Diego, CA) was injected IP 3 times per week (4×10^4 units per injection; see Supplementary Materials and Methods). For cell culture experiments, PGE_2 was added at 25 μM. IL-2 was used at 10 ng/mL.

Preparation of Gastric Tissue and Assessment of Colonization, Gastric IFN-γ Production, COX-2 Expression, Neutrophil Infiltration, and Histopathology

After sacrifice, stomachs were dissected longitudinally into equally sized pieces. For the assessment of *H felis* colonization, total genomic stomach DNA was subjected to quantitative polymerase chain reaction analysis of the *fla*B gene, as described previously.[7] Quantitative analysis of gastric IFN-γ expression and COX-2 expression and activity was performed as described in the Supplementary Materials and Methods. Neutrophil infiltration was quantified as described.[7] Paraffin sections were stained with the histological dyes Giemsa, Alcian Blue, and Periodic Acid Schiff, as well as an antibody specific for proliferating cell nuclear antigen (clone PC10; Zymed Labs, San Francisco, CA) for grading of gastric histopathology based on the features described in the updated Sydney classification (see Supplementary Materials and Methods).[23]

Lymphocyte Proliferation and Activation Assays

$CD4^+CD25^-$ T cells were immunomagnetically purified from single-cell suspensions of spleens, mesenteric lymph nodes, or stomach sections containing both corpus and antral tissue (R&D Systems, Minneapolis, MN). Cells were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3/CD28-coated beads. The CFSE dilution was assessed flow cytometrically. IFN-γ secretion was measured by enzyme-linked immunosorbent assay (BD Biosciences, San Diego, CA). IL-2 and IFN-γ expression was determined by reverse transcription polymerase chain reaction using primers specified in the Supplementary Materials and Methods. For staining of intracellular IFN-γ, cells were stained for CD4 (clone RM4-5; eBioscience, San Diego, CA) prior to fixation, permeabilization, and IFN-γ staining (XMG1.2, BD Biosciences). Fluorescence-activated cell sorting analyses were performed on a CyanADP instrument (Dako, Glostrup, Denmark). T-cell migration assay is described in the Supplementary Materials and Methods.

Statistical Analysis

P values were calculated using Graph Pad prism 5.0 software (GraphPad Software, San Diego, CA). The significance of differences in histopathology scores was

calculated by Mann–Whitney test and the significance of numerical differences was calculated by Student's *t* test. All in vitro assays were analyzed in triplicate and are shown with standard deviations.

Results

Helicobacter *Infection Induces COX-2 and PGE$_2$ In Vitro and In Vivo*

To assess whether COX-2 and its product PGE$_2$ are induced upon *Helicobacter* infection in cell culture models, we infected primary murine gastric epithelial cells (Figure 1*A*) and immortalized murine gastric epithelial cells (Figure 1*B*) with wild-type *H pylori* G27 or an isogenic mutant (ΔPAI) lacking the Cag pathogenicity island, a known virulence determinant of pathogenic *H pylori* strains.[22] In both cell types, COX-2 protein expression and PGE$_2$ secretion were induced by *H pylori* in a dose-dependent manner, and independently of the Cag PAI (Figure 1*A*–*C*). PGE$_2$ production was blocked in the presence of the COX inhibitor indomethacin (Figure 1*C*), indicating that it is produced exclusively by COX enzymes in this setting. COX-2 and its product PGE$_2$ were also strongly induced in the gastric mucosa of infected compared to uninfected mice (Figure 1*D* and *E*).

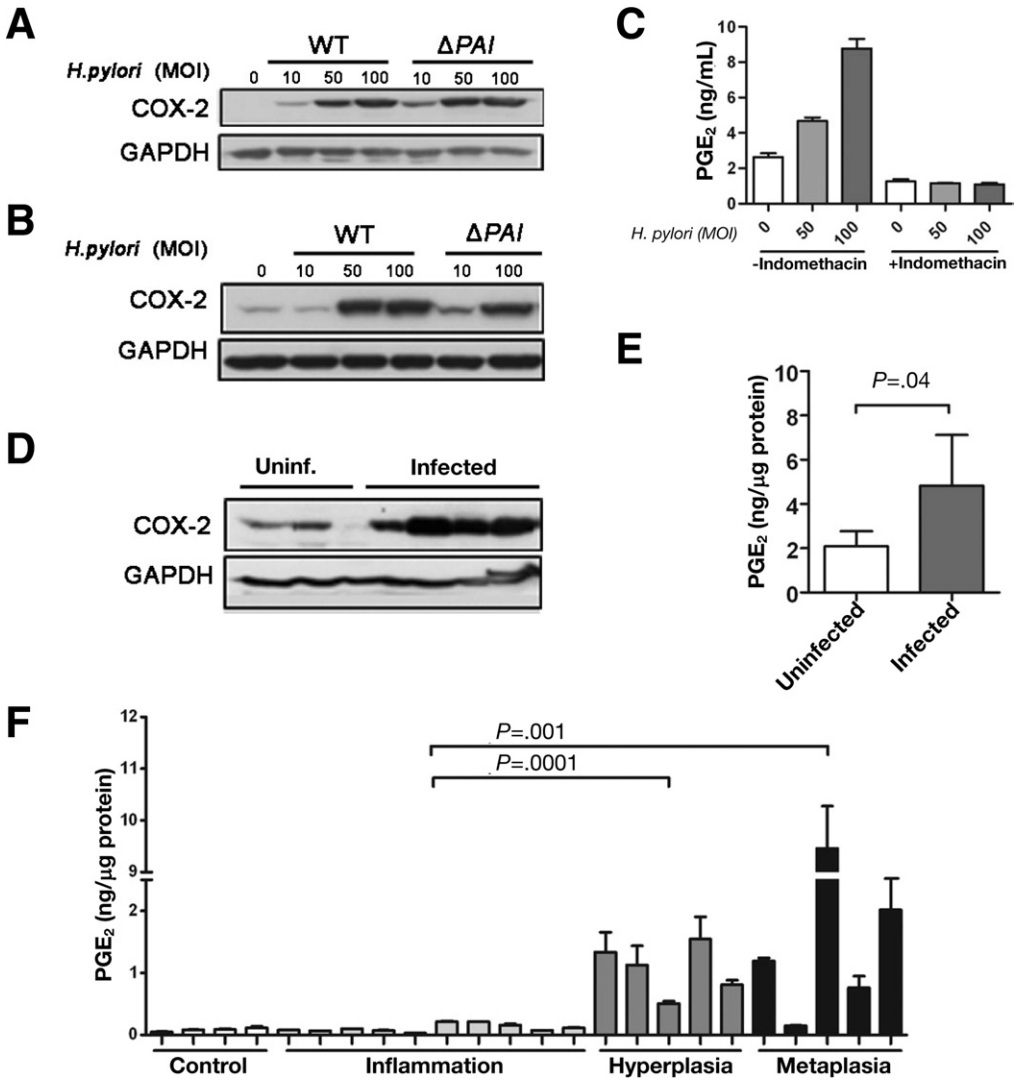


Figure 1. COX-2 protein expression and PGE$_2$ production is induced upon *Helicobacter* infection in vitro and in vivo. COX-2 protein expression was analyzed by Western blotting of extracts from primary murine gastric epithelial cells (*A*) and an immortalized murine gastric epithelial cell line (*B*) infected for 12 hours with either *H pylori* G27 wild-type (WT) or an isogenic Cag-PAI-deficient mutant (ΔPAI) at the indicated multiplicity of infections. Glyceraldehyde phosphate dehydrogenase (GAPDH) levels are shown as loading controls. PGE$_2$ production as assessed by competitive enzyme-linked immunosorbent assay (ELISA) is shown for supernatants of primary cells infected with WT G27, in the presence or absence of 28 μM indomethacin (*C*). For the analysis of COX-2 protein expression and PGE$_2$ production in vivo, whole stomach extracts were generated from four 5-month *H felis*–infected and 3 control C57BL/6 mice and subjected to Western blot analysis (*D*) and PGE$_2$ ELISA (*E*). (*F*) To determine mucosal PGE$_2$ concentrations as a function of pathology, we compared groups that showed gastritis only, hyperplasia, or metaplasia to uninfected controls. In (E) and (*F*), all samples were analyzed in triplicate; average values are shown with standard deviations.

In order to determine whether mucosal PGE_2 concentrations correlate with the degree and type of gastric pathology in our model, we selected groups of *Helicobacter*-infected mice based on their predominant pathology. We compared mice that showed gastritis, hyperplasia, or acidic mucus-positive metaplasia, respectively (for simplicity referred to as "metaplasia" in the following; Figure 1*F*). PGE_2 production was significantly higher in animals displaying epithelial damage, eg, hyperplasia and metaplasia, compared to those that showed gastritis only (Figure 1*F*). Our data are consistent with previous studies reporting COX-2 expression in *H pylori*–infected cultured epithelial cells[24] and in gastric preneoplastic and malignant lesions.[25]

COX-2 Inhibition During Infection Aggravates Helicobacter*-Induced Epithelial Pathology in C57BL/6 Mice*

To test if COX-2 activity modulates the formation of *Helicobacter*-associated chronic gastritis and precancerous lesions, we treated *H felis*–infected mice with celecoxib for 3 months (Figure 2*A*). Celecoxib specifically targets COX-2 and is believed to act predominantly by decreasing PGE_2 levels.[15] The efficacy of celecoxib treatment was confirmed by a COX-2–specific enzymatic activity assay (Figure 2*B*). The inhibition of COX-2 significantly aggravated *H felis*–induced epithelial atrophy and hyperplasia compared to infected, untreated counterparts as determined by quantitative histopathological scoring of histologically stained sections (Figure 2*C*). The more severe pathology observed under conditions of COX-2 inhibition was accompanied by a significant decrease in *H felis* colonization (Figure 2*D*), and a strong increase of the gastric expression of the Th1 cytokine IFN-γ (Figure 2*E*), corroborating both the direct correlation between IFN-γ levels and gastric histopathology, and the inverse correlation between IFN-γ expression and colonization that we have reported previously.[7] In conclusion, COX-2 inhibition accelerates development of *Helicobacter*-induced early gastric cancer precursor lesions, while simultaneously reducing the bacterial burden and increasing the local expression of IFN-γ, implying that COX-2 and PGE_2 may act as immune modulators of the *Helicobacter*–host interaction.

Treatment of Helicobacter*-Infected Mice With Synthetic PGE_2 Inhibits Chronic Inflammation and Precancerous Lesions*

To investigate a possible protective effect of COX-2 activity on the *H felis*–infected gastric mucosa in an independent experimental setup, we treated C57BL/6 mice with synthetic PGE_2 during 6 or 12 weeks of infection (Figure 3*A*). To ensure complete activation of all PGE_2 receptors (E-prostanoid receptors 1–4), we administered a mixture of 2 synthetic PGE_2 analogs, 16,16-dimethyl PGE_2 and 17-phenyl trinor PGE_2 (referred to in the following as "PGE_2"). The PGE_2 treatment prevented the mild to moderate lymphocytic infiltration observed after 6 weeks postinfection (Figure 3*A* and *B*) and also completely inhibited the moderate to severe chronic inflammation, atrophy, metaplasia, and/or hyperplasia indicative of 12-week–infected mice (Figure 3*A* and *C*). Immunohistochemical staining and quantification of proliferating cell nuclear antigen reactivity further confirmed the efficient inhibition of epithelial hyperproliferation by PGE_2 (Figure 3*D*). Consequently, the decrease in *H felis* colonization that typically accompanies the onset of severe gastric pathology between 6 and 12 weeks postinfection was prevented by PGE_2 treatment (Figure 3*E*). Long-term treatment with PGE_2 completely prevented the increase in local IFN-γ production that typically occurs in this time frame (Figure 3*F*) and that precedes the onset of pathology and leads to the observed reduction of the bacterial burden.[7]

We next tested the protective potential of PGE_2 in mice lacking the gene for the anti-inflammatory cytokine IL-10. IL-$10^{-/-}$ mice launch excessive proinflammatory and adaptive immune responses to *Helicobacter* infection;[26] as a result, they clear the bacteria and develop severe gastritis and preneoplastic lesions already 4 weeks postinfection (Figure 4). Treatment of infected IL-$10^{-/-}$ animals with synthetic PGE_2 efficiently inhibited development of gastric pathology and hyperproliferation (Figure 4*A*–***C***) and prevented neutrophil infiltration into the gastric mucosa (Figure 4*D*), but did not block clearance of the infection (data not shown). As in wild-type mice, local induction of IFN-γ was largely prevented by PGE_2 treatment in IL-$10^{-/-}$ mice (Figure 4*E*). The combined results indicate that prophylactic PGE_2 treatment efficiently inhibits development of chronic gastritis and cancer precursor lesions, possibly by modulating Th1-polarized T-cell responses.

Preexisting Gastric Cancer Precursor Lesions in H felis*–Infected Mice Can Be Reversed by PGE_2 Treatment*

To test the effects of PGE_2 as a potential therapeutic agent capable of curing preexisting lesions, we infected mice with *H felis* for 3 months and verified the efficient induction of preneoplastic pathology in a small control group (data now shown). Five of the remaining 13 mice received PGE_2 for another month (Figure 5*A*). Interestingly, this treatment efficiently reversed preexisting lesions, reducing the scores of all histopathological parameters (Figure 5*A* and *B*) and the fraction of corpus area affected by proliferating cell nuclear antigen–positive hyperplasia (Figure 5*C*), as well as neutrophil infiltration (Figure 5*D*). As in prophylactically treated mice, PGE_2 had a beneficial, in this case even curative, effect on mucosal integrity, while at the same time boosting bacterial colonization (Figure 5*E*). In line with all previous results (Figures 3 and 4), the PGE_2 treatment significantly reduced local gastric expression of IFN-γ (Figure 5*F*).

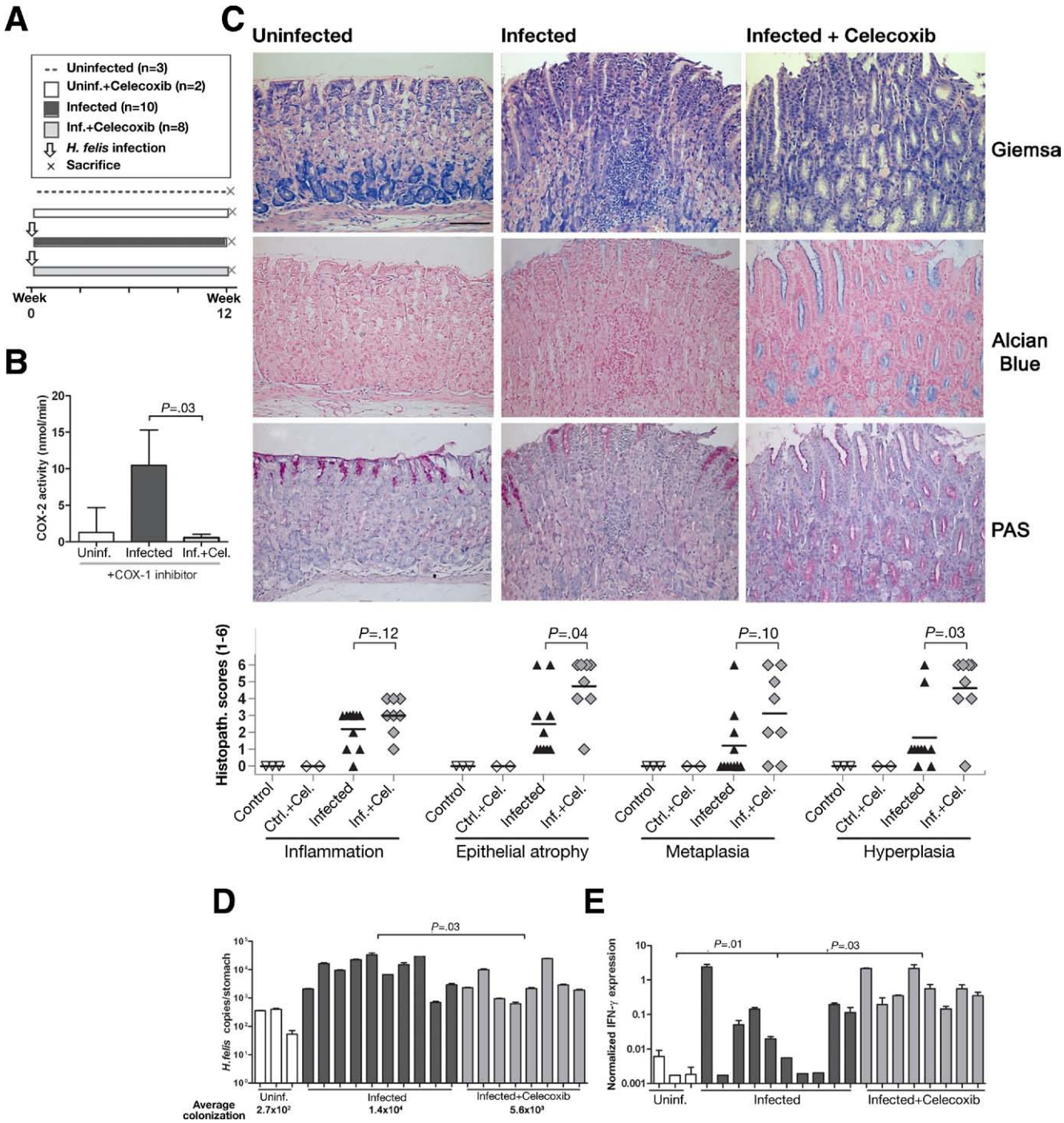


Figure 2. Inhibition of COX-2 by the diet-supplemented inhibitor celecoxib aggravates gastritis and gastric preneoplastic pathology induced by *H felis* infection. C57BL/6 mice were infected for 3 months with *H felis,* or remained uninfected, and were either fed a control diet or a diet supplemented with celecoxib (*A*). COX inhibition was verified by COX activity assay (*B*). All mice were analyzed with respect to gastric histopathology (representative Giemsa-, Alcian Blue-, and Periodic Acid Schiff–stained sections and pathology scores are shown. (*C*) *H felis* colonization (*D*) the average colonization is indicated per group and gastric expression of IFN-γ (*E*). Scale bar = 50 μm.

To confirm regression of preexisting lesions upon PGE$_2$ treatment during the last of 4 months of *H felis* infection, we repeated the experiment with 3 additional readouts (Supplementary Figure 1). As before, the lesions regressed in all PGE$_2$-treated mice compared to the infected control group (data not shown); we further found by flow cytometry that the gastric infiltration of leukocytes and CD4$^+$ T cells had significantly decreased in the PGE$_2$-treated group (Supplementary Figure 1*A* and *B*). Finally, we examined the presence of Th1-polarized, IFN-γ–producing–CD4$^+$ T cells in the mesenteric lymph nodes (MLN) by intracellular cytokine staining followed by fluorescence-ac-

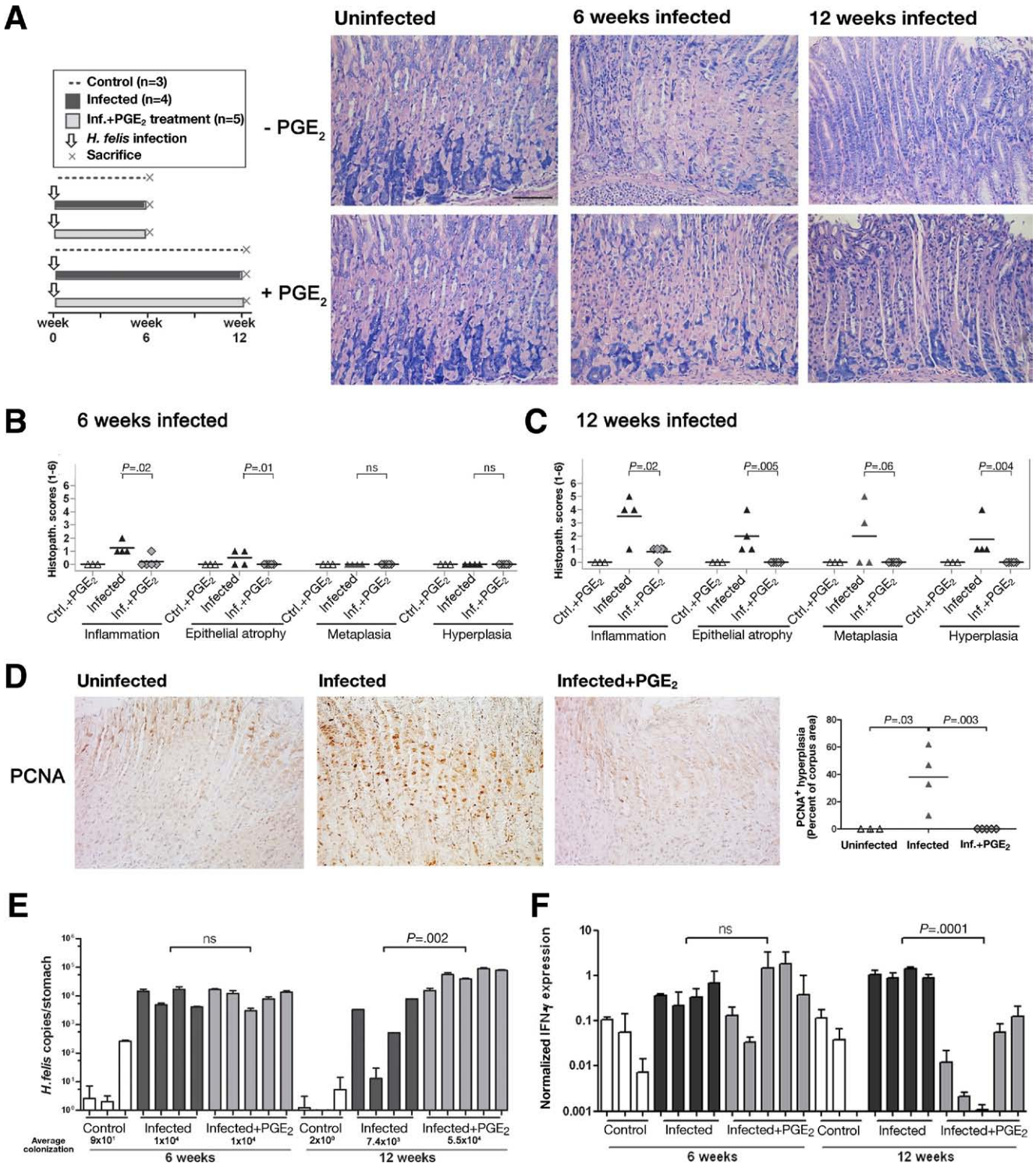


Figure 3. PGE_2 treatment of *H felis*–infected C57BL/6 wild-type mice inhibits chronic inflammation and epithelial pathology. C57BL/6 mice were infected with *H felis* and were treated with PGE_2 as indicated (*A*). Mice were euthanized 6 weeks (*A, B, E, F*) or 12 weeks postinfection (*A, C, E, F*). Representative Giemsa-stained sections and a summary of all histopathology scores are shown in (*A–C*); representative proliferating cell nuclear antigen (PCNA)–stained sections after 12 weeks of infection, along with their quantification, are shown in (*D*). *H felis* colonization and gastric IFN-γ expression are shown in (*E*) and (*F*).

tivated cell sorting. We found that the 4-month infection indeed led to a strong increase of IFN-γ–producing $CD4^+$ T cells in the MLN, which could be blocked at least partially by PGE_2 treatment (Supplementary Figure 1*C*). We conclude from both studies that systemically administered PGE_2 efficiently reverses preexisting gastric cancer precursor lesions, possibly by blocking the priming and activity of Th1-polarized T-cell responses in the draining lymph nodes.

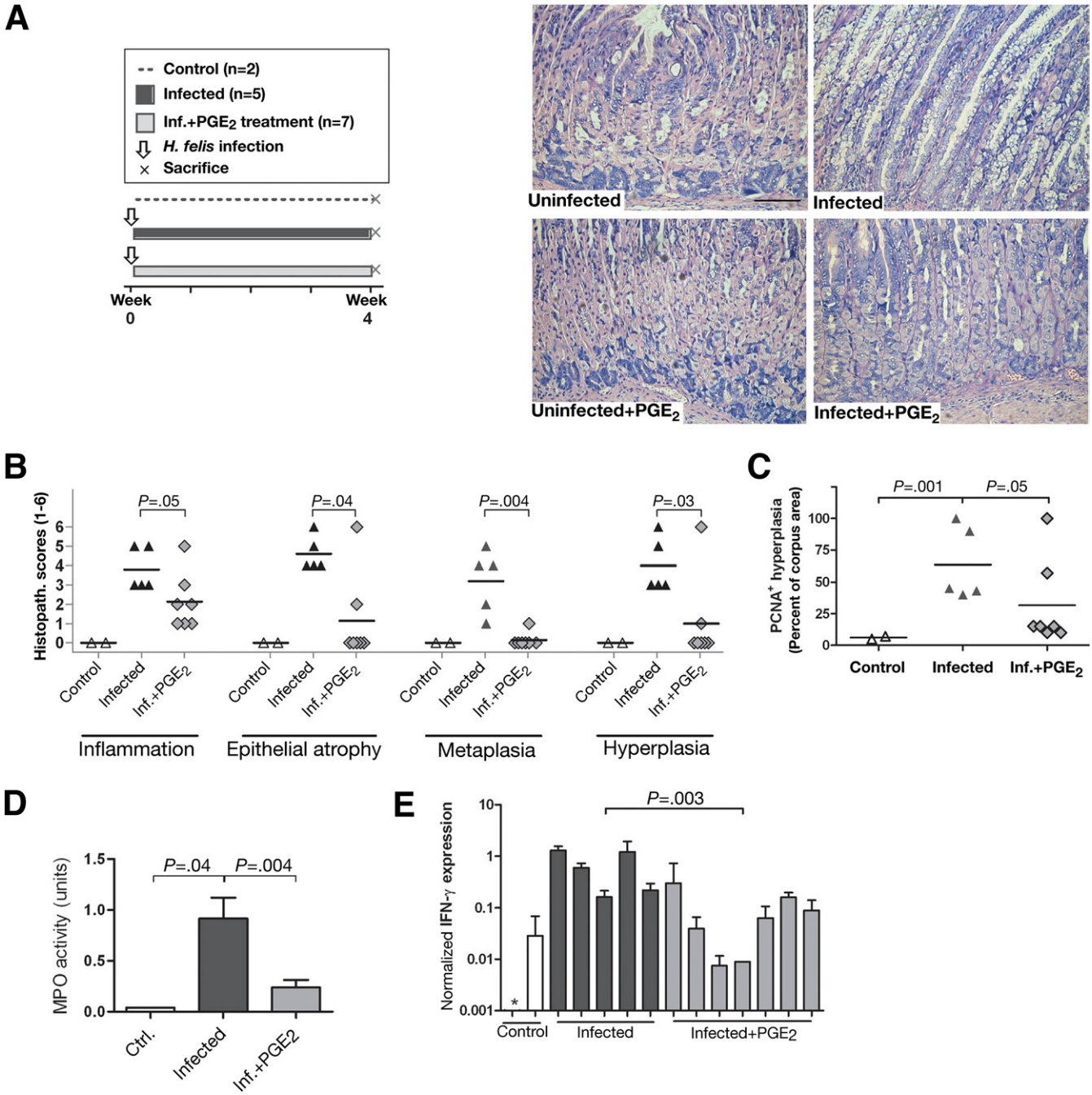


Figure 4. PGE$_2$ treatment of *H felis*–infected IL-10$^{-/-}$ BL/6 mice inhibits chronic inflammation and epithelial pathology IL-10$^{-/-}$ BL/6 mice were infected for 1 month with *H felis* and were treated with PGE$_2$ starting on the day of infection (*A*). Representative Giemsa-stained sections and histopathology scores are shown in (*A*) and (*B*); the quantification of proliferating cell nuclear antigen (PCNA)–positive hyperplasia is depicted in (*C*). Neutrophil infiltration was quantified by myeloperoxidase activity assay [averages of all mice per group are shown with standard deviations, (*D*)], and gastric IFN-γ expression was assessed by real-time polymerase chain reaction (*E*); (*not detectable).

PGE$_2$ Impairs Vaccine-Induced Protection Against Helicobacter *by Blocking Local Effector T-Cell Responses*

We have shown in the experiments outlined here that PGE$_2$ treatment in vivo boosts bacterial colonization and, conversely, that COX-2 inhibition promotes clearance (Figures 2*D*, 3*E*, and 5*E*). To test whether PGE$_2$ would also impair vaccine-induced protection upon challenge infection of immunized mice, we utilized a vaccination protocol directed against *H pylori* SS1. Indeed, administration of PGE$_2$ during challenge impaired the vaccine-induced reduction of bacterial loads (Figure 6*A*). The infiltration of leukocytes and CD4$^+$ T cells into the gastric mucosa observed in vaccinated/challenge-infected

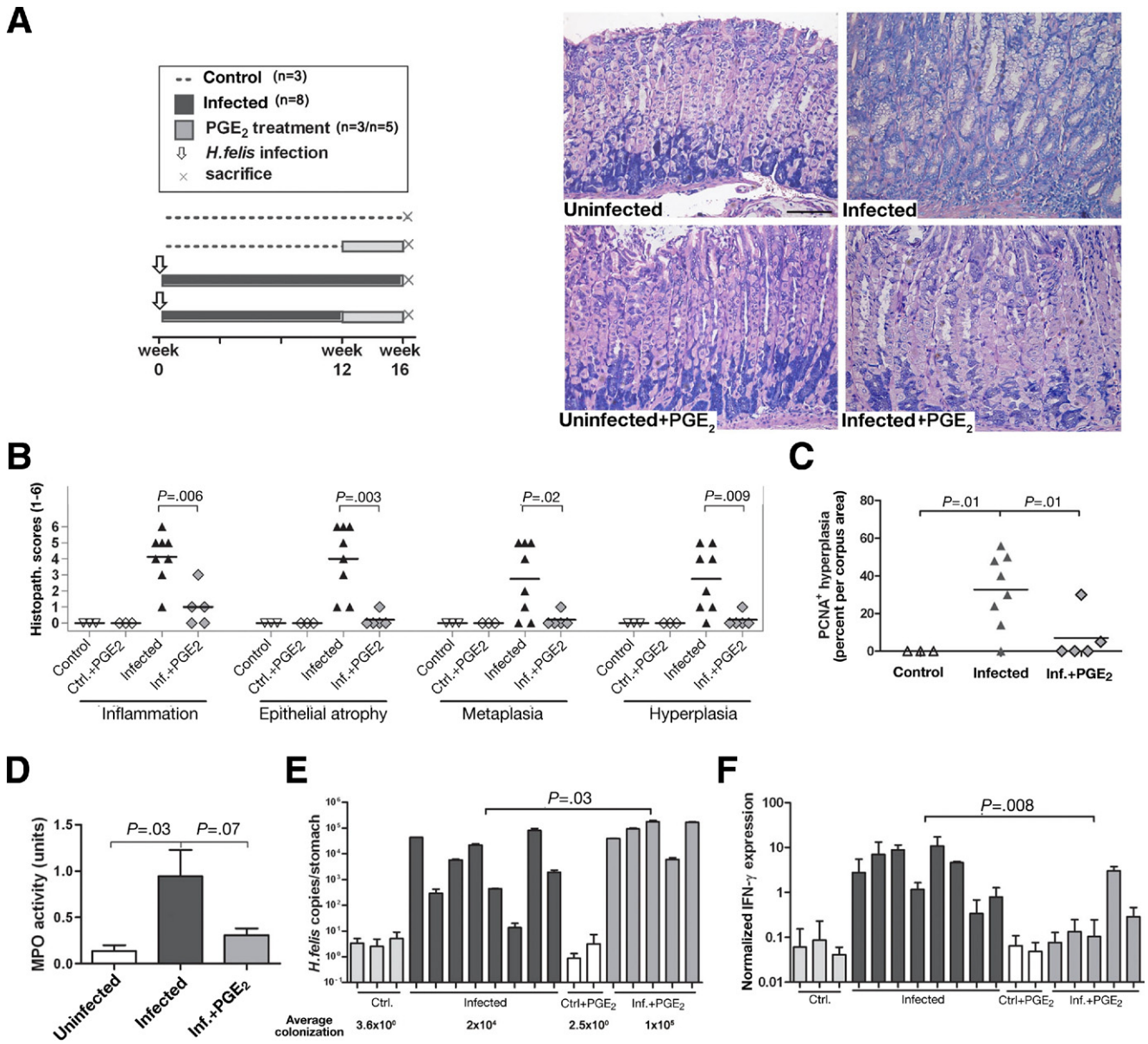


Figure 5. PGE_2 treatment of C57BL/6 mice reverses preexisting gastric precursor lesions. C57BL/6 mice were infected for a total of 16 weeks with *H felis* and treated with PGE_2 for the last month as indicated (*A*). Representative Giemsa-stained sections and histopathology scores are shown in (*A*) and (*B*); the quantification of proliferating cell nuclear antigen (PCNA)–positive hyperplasia in percent of the corpus area is depicted in (*C*). (*D*) Neutrophil infiltration was quantified by myeloperoxidase activity assay. *H felis* colonization and gastric interferon-γ expression are shown in (*E*) and (*F*).

mice was largely prevented by administration of PGE_2 (Figure 6*B* and *C*), possibly explaining why PGE_2-treated mice fail to reduce bacterial colonization. In single-cell MLN preparations of individual mice (Figure 6*D*), PGE_2 treatment during the challenge phase clearly abolished the vaccination-induced increase of IFN-γ–producing $CD4^+$ T cells in the MLN (Figure 6*D*). Taken together, our data imply that PGE_2 interferes with the T-cell–mediated control of *Helicobacter* infection, not only under standard experimental infection conditions, but also during a challenge infection after vaccination, and that this effect is due to T-cell inhibition in vivo.

To test whether PGE_2 directly interferes with IFN-γ production by MLN cells, single-cell suspensions were cultured in the presence of increasing concentrations of PGE_2 (Figure 6*E*). PGE_2 treatment reduced the infection-dependent secretion of IFN-γ into the supernatant by, on average, 43% and 94% at a low and high dose, respectively (Figure 6*E*). We conclude that PGE_2 efficiently blocks production of IFN-γ both in vitro and in vivo, which may explain why PGE_2-treated mice are protected from IFN-γ–induced preneoplastic pathology and show impaired bacterial clearance, a finding that is consistent with our previous report of an inverse link between colonization and pathology.[7]

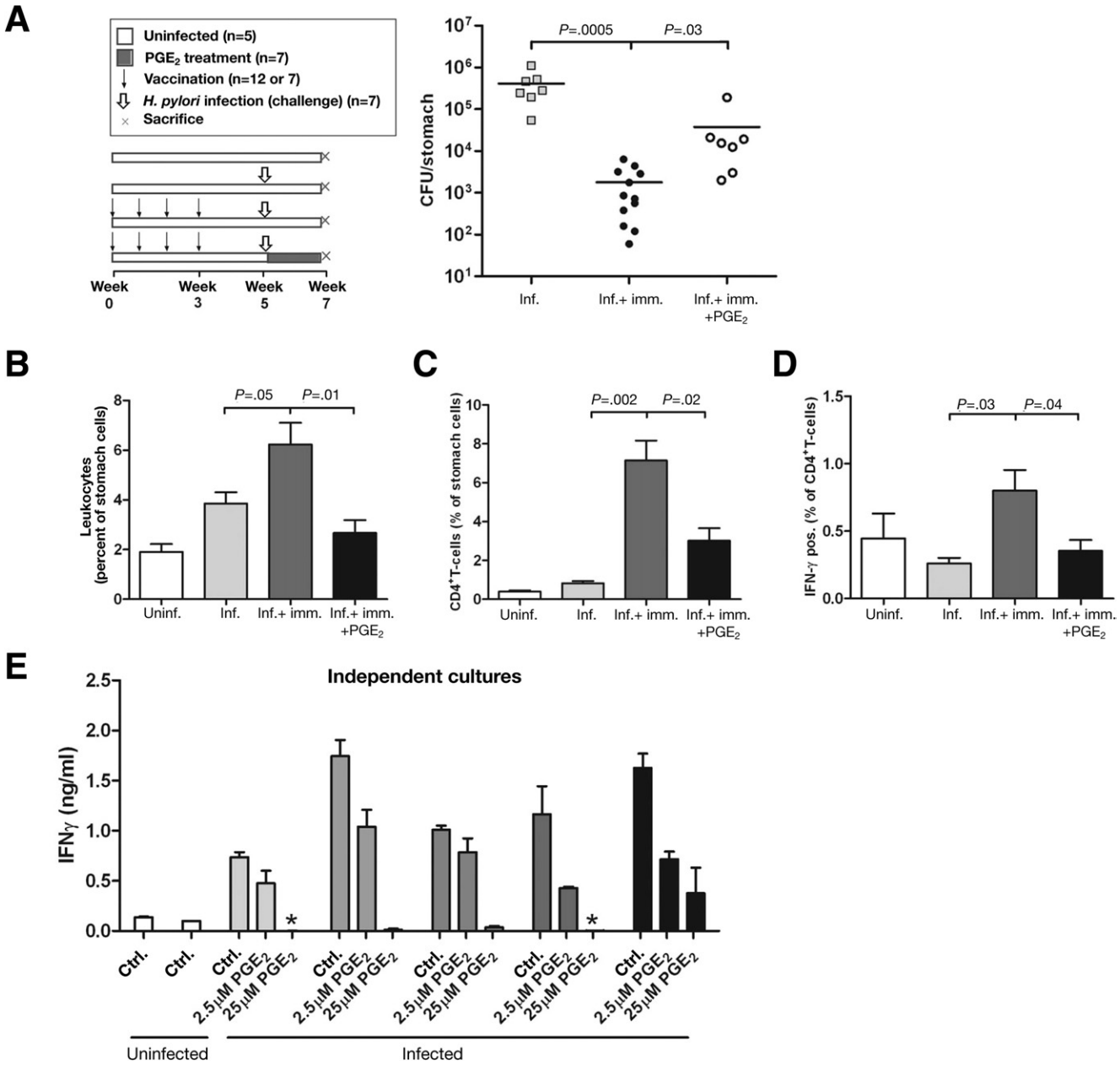
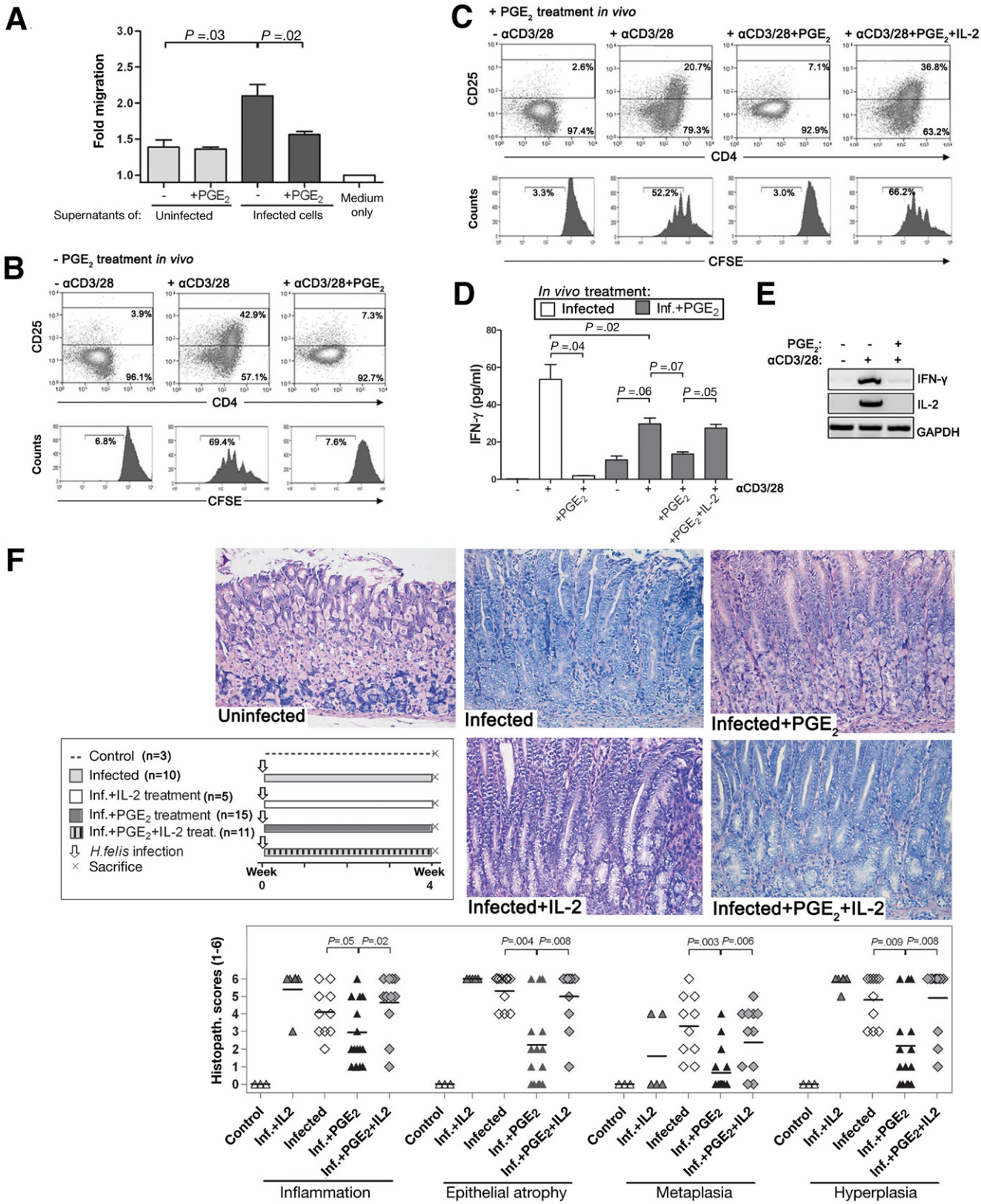


Figure 6. PGE$_2$ treatment during challenge infection impairs vaccine-induced clearance. C57BL/6 mice were vaccinated 4 times and challenged with live *H pylori* SS1; some mice received PGE$_2$ during the challenge phase as indicated. Nonvaccinated infected mice served as controls (*A*). Colony counts are shown as colony-forming unit (CFU)/stomach (*A*). (*B–D*) Single-cell suspensions from stomachs (*B, C*) and MLN (*D*) were generated for 2 uninfected, 4 control infected, 4 immunized/challenged, and 4 immunized/challenged/PGE$_2$-treated mice and analyzed individually by flow cytometry. Leukocyte (*B*) and CD4$^+$ T-cell (*C*) infiltration into the stomach is shown in percent of total stomach cells. (*D*) The IFN-γ–producing CD4$^+$ T-cell population in the MLN was quantified by intracellular cytokine staining. Averages and standard deviations are shown for every treatment group. (*E*) Single-cell MLN cultures obtained from 5 *H felis*–infected and 2 control mice were cultured overnight in the presence or absence of PGE$_2$ (2.5 μM or 25 μM). IFN-γ secretion into the supernatants was assessed by enzyme-linked immunosorbent assay (*not detectable).

PGE$_2$ Inhibits the Migration, Activation, and Effector Functions of Wild-Type CD4$^+$ T Cells

To test the effects of PGE$_2$ on CD4$^+$CD25$^-$ T-cell migration, we utilized a transwell migration assay in which calcein-labeled CD4$^+$CD25$^-$ T cells were allowed to migrate toward supernatants of *H pylori*–infected immortalized gastric epithelial cells. Interestingly, the supernatants of infected cells attracted T cells more efficiently than those of uninfected cells (Figure 7*A*). The migration could be blocked by addition of PGE$_2$ (Figure 7*A*). In order to assess the effects of PGE$_2$ on the proliferation and activation of CD4$^+$ effector T cells, we stimulated CFSE-labeled CD4$^+$CD25$^-$ T cells with anti-CD3/anti-CD28 monoclonal antibody–coated beads in the presence or absence of PGE$_2$ (Figure 7*B*). The activation


A
Fold migration
P =.03
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Supernatants of:
Uninfected
Infected cells
Medium only
B
- PGE2 treatment in vivo
- αCD3/28
+ αCD3/28
+ αCD3/28+PGE2
CD25
CD4
Counts
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C
+ PGE2 treatment in vivo
+ αCD3/28+PGE2+IL-2
D
In vivo treatment:
Infected
Inf.+PGE2
IFN-γ (pg/ml)
E
PGE2:
αCD3/28:
IFN-γ
IL-2
GAPDH
F
Uninfected
Infected
Infected+PGE2
Infected+IL-2
Infected+PGE2+IL-2
Control (n=3)
Infected (n=10)
Inf.+IL-2 treatment (n=5)
Inf.+PGE2 treatment (n=15)
Inf.+PGE2+IL-2 treat. (n=11)
H.felis infection
Sacrifice
Week 0
Week 4
Histopath. scores (1-6)
Inflammation
Epithelial atrophy
Metaplasia
Hyperplasia

and proliferation of T cells, as assessed by measuring IL-2 receptor α chain (CD25) expression and CFSE dilution, was blocked almost completely by PGE_2 (Figure 7*B*). In vivo pretreatment of 5 donor mice with PGE_2 for 1 month prior to T-cell isolation also strongly reduced T-cell activation and proliferation upon CD3/CD28 cross-linking (compare Figure 7*B* and *C*). The secretion of IFN-γ by CD3/CD28-activated T cells was also blocked by PGE_2, both by the direct addition of the compound to the cultures and by pretreatment of the donor mice (Figure 7*D*). These results suggest that PGE_2 is an effective immunosuppressant capable of blocking the activation and proliferation of $CD4^+$ T cells, as well as their ability to secrete IFN-γ. $CD4^+$ effector T-cell activation and proliferation requires an autocrine-positive feedback loop of IL-2 secretion and IL-2 receptor activation.[27] We, therefore, asked whether the first step in this cascade, the transcriptional activation of the *IL-2* gene, could be blocked by PGE_2. Indeed, PGE_2 treatment efficiently inhibited *IL-2* and *IFNG* gene expression induced by CD3/CD28 cross-linking (Figure 7*E*). Therefore, we tested whether adding recombinant IL-2 would reverse the effects of PGE_2. Indeed, addition of IL-2 was able to completely reverse the effects of PGE_2 on T-cell activation, proliferation, and IFN-γ secretion (Figure 7*C* and *D*). These results suggest that PGE_2 inhibits T cells, at least in part, by interfering with their autocrine IL-2–driven positive feedback loop.

Based on studies reporting that PGE_2 may act as an immunosuppressant by stimulating the suppressive activities of regulatory T cells (Tregs),[28] we speculated that Tregs might be an additional target of PGE_2 in the control of T-cell–mediated immunopathology in the *Helicobacter*-infected stomach. To examine this possibility, we preconditioned immunomagnetically isolated $Ly5.2^+CD4^+CD25^+$ T cells with PGE_2 for 6 hours prior to coculturing them with CFSE-labeled $Ly5.1^+$ effector T cells at various ratios. Indeed, we found that PGE_2 pretreatment modestly enhanced the suppressive capacity of Tregs, albeit only at a ratio of suboptimal suppression (Supplementary Figure 2). This piece of data suggests that Tregs can indeed respond to PGE_2 in vitro and become more suppressive as a result of PGE_2 stimulation.

Recombinant IL-2 Reverses the Effects of PGE$_2$ In Vivo

In order to investigate whether the observed opposing effects of IL-2 and PGE_2 hold true in vivo, and to explore whether T cells are indeed critical targets of PGE_2, we utilized the previously mentioned $IL\text{-}10^{-/-}$ model (Figure 4). In addition to a group treated with PGE_2 only, we also included groups that received IP doses of recombinant IL-2 (4×10^4 units, 3 times weekly) together with PGE_2 or IL-2 alone (Figure 7*F*). IL-2 administration efficiently reversed the protection from gastric pathology conferred by PGE_2 (Figure 7*F*). This result suggests that the protective effect of PGE_2 on the infected gastric mucosa is due to the suppression of T-cell effector functions, which are known to directly trigger the atrophic gastritis, hyperplastic, and metaplastic transformation that are a hallmark of the infection in mice as well as in humans.[7]

Discussion

In this study, we provide experimental evidence that COX-2/PGE_2-dependent pathways play an important immunomodulating role during the early stages of *Helicobacter*-induced gastric pathogenesis, thereby preventing excessive gastric immunopathology. We show that systemic administration of PGE_2 prevented, and COX-2 inhibition exacerbated, *Helicobacter*-induced atrophic gastritis, epithelial hyperplasia, and metaplasia. These effects were due to the immunosuppressive effects of PGE_2 on T cells, which failed to proliferate and to secrete IL-2 and IFN-γ when exposed to PGE_2 in vitro or in vivo.

Our data are in line with several previous studies reporting exacerbated gastritis in *H pylori*–infected rodents under conditions of COX-1 or -2 inhibition or deletion of the *COX-1* or *-2* genes.[29–32] Similar effects were observed in human trials investigating the effects of COX inhibi-

Figure 7. PGE_2 treatment inhibits $CD4^+CD25^-$ T-effector cell migration, activation, proliferation, and IFN-γ secretion in vitro, and recombinant IL-2 reverses the effects of PGE_2 in vivo. (*A*) 1×10^5 Calcein-labeled splenic $CD4^+CD25^-$ T cells were seeded into transwell chambers and allowed to migrate toward supernatants of *H pylori*–infected or uninfected immortalized gastric epithelial cells in the presence or absence of 25 μM PGE_2. The migration was quantified spectrophotometrically; data are displayed with the baseline migration of cells toward unconditioned medium set as 1. (*B–E*), $CD4^+CD25^-$ cells were isolated from the combined MLN of 5 infected animals (*B,D,E*) or of 5 infected animals that had been pretreated with PGE_2 in vivo for 1 month (*C,D*). Cells were labeled with CFSE and stimulated with αCD3/28-coated beads; 25 μM PGE_2 and/or 10 ng/mL IL-2 was added where indicated. The CFSE dilution as a measure of proliferation (*lower panels* of *B,C*) and CD25 up-regulation as a measure of activation (*upper panels* of *B,C*) were assessed flow-cytometrically. All culture conditions were analyzed in triplicate; all statistical comparisons [$\pm$anti-CD3/CD28, $\pm PGE_2$, IL-2 in (*B*) and (*C*); + anti-CD3/CD28 with or without PGE_2 pretreatment in vivo] revealed significant differences ($P \leq .05$) for the readouts of CFSE dilution and of CD25 up-regulation. (*D*) The anti-CD3/CD28-induced secretion of IFN-γ into the culture supernatants was measured by enzyme-linked immunosorbent assay for the same cultures as shown in (*B*) and (*C*). (*E*) IL-2 and IFN-γ expression was assessed by semi-quantitative reverse transcription polymerase chain reaction. A representative experiment of 3 (*A*) and 4 (*B–E*) is shown. (*F*) $IL10^{-/-}$ BL/6 mice were infected for 1 month with *H felis* and treated with PGE_2 or recombinant IL-2 or a combination of the 2. Representative Giemsa-stained sections and histopathology scores are shown. No significant differences were observed between IL-2–treated groups $\pm$ PGE_2.

tion by aspirin in *H pylori*–infected, asymptomatic individuals, who suffered significantly more from mucosal injury and peptic ulcers than their uninfected counterparts.[33,34]

Despite the well-documented side effects of aspirin use on the gastric mucosa, the long-term, low-level use of aspirin or nonaspirin nonsteroidal anti-inflammatory drugs prevents development of gastric cancer; this association holds true even in a large meta-analysis including a total of 9 studies.[14] Similarly, treatment with the COX inhibitor, nimesulide, prevented a substantial portion of chemically induced gastric cancers in *H pylori*–infected mice.[35] The chemopreventive outcome of COX inhibition in studies examining cancer as the end point is generally believed to be due to the powerful effects of tumor-derived PGE_2 on cancer cell survival and invasion, tumor-associated angiogenesis, and on anti-tumor immunosuppression, all of which promote cancer progression.[15]

We demonstrate here that T cells, in particular effector subsets of the T-helper compartment, can be targeted by PGE_2. We have shown in a previous study that *Helicobacter*-induced gastric preneoplastic lesions result from diffuse infiltration of $CD4^+$, Th1-polarized effector T cells into the gastric lamina propria, where T-cell–derived IFN-γ acts directly on gastric epithelial lineages to promote their hyperproliferation and their transformation to intestinal-type cells.[7] Effector functions of $CD4^+$ T cells, such as proliferation, IFN-γ production, and migration are blocked upon exposure to PGE_2 in our models, with the underlying mechanism being a block of *IL-2* gene transcription. Consequently, administration of recombinant IL-2 was sufficient to override the protective effects of synthetic PGE_2 in vitro and in vivo. The profound effect of PGE_2 on $CD4^+$ T-cell effector functions, maintained even in explanted T cells restimulated ex vivo, also explains why PGE_2-treated mice are colonized at higher levels than untreated counterparts, and show impaired vaccine-induced protection. We conclude that PGE_2 averts immunopathology in the infected stomach by T-cell suppression, the necessary consequence of which is persistent colonization with *Helicobacter*. In seeming contrast to the protective effect of exogenously added PGE_2, we observe elevated endogenous PGE_2 in mice with established atrophy. We believe that this PGE_2, which may be COX-1–derived, is secreted by the gastric mucosa in an effort to repair the mucosal damage,[36] but is insufficient to act on T cells and to prevent further damage or to reverse preestablished lesions.

Our finding that PGE_2 treatment protects IL-$10^{-/-}$ mice from gastric pathology argues against Tregs being the primary target of PGE_2-driven immunosuppression as IL-$10^{-/-}$ Tregs are incapable of blocking the *Helicobacter*-associated gastritis induced by adoptive transfer of effector T cells into infected Rag-$2^{-/-}$ recipients.[37] However, we did observe that Tregs became modestly more suppressive in vitro upon pretreatment with PGE_2, as demonstrated previously by Sharma et al;[28] this observation suggests that the combined effects of PGE_2 on effector and regulatory T cells may together account for the complete block in T-cell–driven immunopathology observed in our wild-type infection models.

An intriguing finding of our study was the observed therapeutic effect of PGE_2 on preexisting lesions, an observation that is seemingly incongruent with studies reporting the prevention of gastric cancer by COX inhibition.[14,35] However, it has to be stressed that *Helicobacter*-associated preneoplastic lesions in our mouse models are benign and can be at least partially reversed upon antibiotic eradication of the infection.[38] Under these circumstances, the beneficial effect of PGE_2's suppression of pathogenic T cells apparently outweighs the potential pro-carcinogenic effects that PGE_2 may have on gastric epithelial cells themselves.[32]

In conclusion, our data are consistent with a protective role for PGE_2 during the early stages of the *Helicobacter*-induced gastric carcinogenesis sequence; at the molecular level, PGE_2 blocks the autocrine IL-2–driven positive feedback loop to prevent T-cell effector functions that are required for both *Helicobacter* clearance and induction of preneoplastic gastric immunopathology.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.12.006.

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Received June 16, 2009. Accepted December 8, 2009.

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Acknowledgments

The authors wish to thank Esther Kohler for expert technical assistance. They are grateful to Dirk Bumann, Christoph Dehio, Michael Hottiger, Josef Jiricny, and Isabelle Arnold for helpful discussions. Gregor Fischer is thanked for advice with veterinary issues. The authors are grateful to Stanley Falkow for critical comments on the manuscript.

Conflicts of interest

The authors disclose no conflicts.

Funding

This study was funded by grants from the Swiss National Science foundation, the UBS foundation (BA29 S8Q7-DZZ 969/A), the Swiss Cancer League (OCS-02099-08-2007) and the Nils and Desiree Yde foundation to A.M. Additional funding was supplied by the University Research Priority Program in Systems Biology/Functional Genomics.

Supplementary Materials and Methods

Assessment of Gastric Histopathology

Paraffin sections were stained with the histological dyes Giemsa, Alcian Blue, and Periodic acid Schiff, as well as an antibody specific for proliferating cell nuclear antigen (clone PC10; Zymed Labs) for grading of gastric histopathology based on the features described in the updated Sydney classification.[1] We divided each of the 3 categories of the Sydney system (mild, moderate, and severe) into 2 subcategories as proposed by Chen et al,[2] resulting in a scale of 0–6 (none, 0; mild, 1–2; moderate, 3–4; severe, 5–6). The definitions of the 4 histopathological parameters scored were the following: chronic inflammation: infiltration of the mucosa and submucosa of the antrum and corpus of the stomach with B- and T-lymphocytes; atrophy: loss of specialized, terminally differentiated cells of the gastric mucosa, such as parietal, chief and mucus-producing cells (evident histologically as loss of surface staining with the Periodic Acid Schiff dye); hyperplasia: hyperproliferation of progenitor cells to compensate for the loss of differentiated gastric cells (therefore, sometimes called compensatory epithelial hyperplasia; detected by proliferating cell nuclear antigen reactivity); acidic mucus-producing metaplasia: appearance of acidic mucus-producing goblet-like cells in the gastric corpus mucosa, for simplicity reasons here referred to as metaplasia (histologically evident due to Alcian Blue positivity).

Quantification of COX-2 Expression and Enzymatic Activity, and PGE_2 Levels in Gastric Tissues

COX-2 expression was assessed by Western blotting (no. 160106; Cayman Chemicals, Ann Arbor, MI); COX-2 activity was determined using the COX activity Assay Kit (no. 760151; Cayman Chemicals) according to manufacturer's protocol with addition of COX-1 inhibitor. PGE_2 was assessed by competitive immunoassay (Assay Designs, Ann Arbor, MI) according to manufacturer's instructions.

Pharmacological Treatments (Extended Version)

For inhibition of COX-2, mice were fed a diet supplemented with 1500 ppm celecoxib (D04090202) as described by Jacoby et al[3] (Research Diets, New Brunswick, NJ) or an identical control diet (AIN 76A D1000) without the active compound. The average daily drug dose per mouse was determined to be 3.7 mg, based on the quantification of food intake per cage. For PGE_2 treatment in vivo and in vitro, a stock containing 1 mg/mL 16,16-dimethyl PGE_2 and 1 mg/mL 17-phenyl trinor PGE_2 was prepared in 100% ethanol, as described by Hansen–Petrik et al.[4] To this end, the methyl acetate in which PGE_2 is commercially available had to be vacuum-evaporated and then reconstituted in ethanol. The stock solution was diluted to 10 μg/100 μL in phosphate-buffered saline (PBS) and injected twice IP and once combined IP/orogastrically per week resulting in a total dose of 30 μg/week/mouse. Control mice were injected with PBS. For IL-2 treatment (BD Pharmingen; 50069, Lot 06498) in vivo, we administered 4×10^4 units per injection as proposed by Chong.[5] Recombinant IL-2 was diluted in PBS and administered 3 times weekly IP.

Vaccination of Mice, Challenge Infection, and Colony Count Assay

For vaccination, mice were gavaged 4 times at weekly intervals with 1 mg whole-cell sonicate of *H pylori* strain SS1 together with 10 μg cholera toxin as adjuvant (List Biologicals, Campbell, CA) prior to challenge infection with 10^8 colony-forming unit *H pylori* SS1. Mice were sacrificed 2 weeks postchallenge; bacterial colonization was assessed by plating of increasing volumes (1, 10, and 100 μL) of homogenized stomach extract on horse blood agar plates followed by colony counting after 5 days of culture under microaerophilic conditions as described previously.[6]

Determination of IL-2 and IFN-γ Expression by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Real-Time RT-PCR

IL-2 and IFN-γ expression were analyzed by RT-PCR using the following primers: IL-2 forward: 5′GCGCACCCACTTCAAGCT 3′; IL-2 reverse: 5′CCTGCTTACAACACATAAGGC3′; IFN-γ forward: 5′GGTGACATGAAA ATCCTGCAGAGC3′; IFN-γ reverse: 5′ TCAGCAGCGACTCCTTTTCCGCTT 3′. The quantitative analysis of gastric IFN-γ expression was performed as described;[6] in brief, total RNA was isolated from scraped gastric mucosa using RNeasy Mini columns (Qiagen, Hilden, Germany). The corresponding complementary DNA served as template for real-time PCR (LightCycler; Roche, Mannheim, Germany) using the LightCycler 480 SYBR Green I master kit (Roche); glyceraldehyde phosphate dehydrogenase expression was determined for normalization.

Preparation of Protein Extracts for COX-2 and PGE_2 Quantification

Protein extracts of scraped gastric mucosa were prepared by homogenization in extraction buffer (20 mM HEPES, 0.4 mM NaCl, 25% glycerol, 1 mM EDTA, 1 mM NaF, and 0.1% NP-40, supplemented with protease inhibitors); 10 μg/mL indomethacin (a COX inhibitor; Sigma, St Louis, MO) was added to all extracts intended for PGE_2 quantification.

Transwell Migration Assay

$CD4^+CD25^-$ T-cells were generated as described here and labeled with calcein (BD Pharmingen); 10^5 lymphocytes were seeded onto 96-well format transwell

plates (ChemoTx Disposable Chemotaxis System, Neuro Probe, Gaithersburg, MD) and chemoattracted by 30 μL conditioned medium for 2 hours at 37°C. The proportion of migrated cells was quantified spectrophotometrically.

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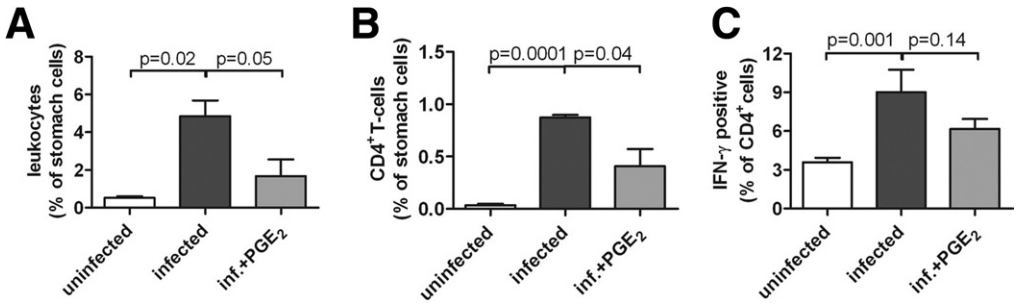
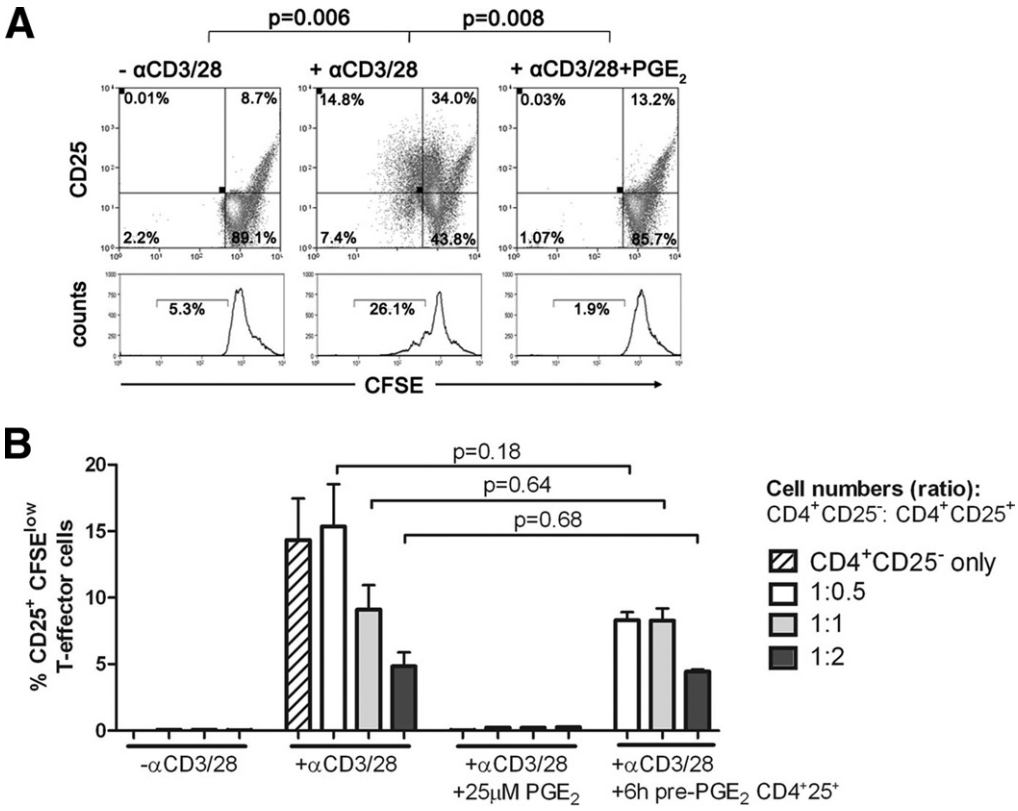


Supplementary Figure 1. Therapeutic PGE_2 treatment reduces the numbers of stomach-infiltrating leukocytes and $CD4^+$ T cells and blocks IFN-γ production in the MLN. C57BL/6 mice were infected for 3 months with *H felis* or remained uninfected (n = 5). A subgroup was treated with PGE_2 during the 4th month of infection (n = 5), whereas the remaining infected mice were left untreated (n = 5). Single-cell suspensions were generated from the stomach and MLN of every mouse and analyzed individually. The fraction of infiltrating leukocytes (A) and $CD4^+$ T-cells (B) in the stomachs and the fraction of $CD4^+$ IFN-γ^+ cells in the MLN (*C*) are shown. Averages and standard deviations were calculated per group.



Supplementary Figure 2. PGE_2 treatment moderately enhances the suppressive activity of $CD4^+CD25^+$ T cells on T-effector cell proliferation. $CD4^+CD25^-$ (Ly5.1BL/6) and $CD4^+CD25^+$(Ly5.2BL/6) T cells were immunomagnetically isolated from the MLN of uninfected mice. 1.5×10^5 CFSE–labeled $CD4^+CD25^-$ cells were stimulated with anti-CD3/CD28-coated beads in the presence or absence of 25 μM PGE_2 (*A*) or combined at increasing ratios with $CD4^+CD25^+$ Treg as indicated (*B*). PGE_2 was either added to combined cultures, or Treg were pretreated for 6 hours with PGE_2 and then added to $CD4^+CD25^-$ cells. Proliferation (CFSE dilution) and activation (CD25 expression) of T-effector cells were analyzed after 3 days by fluorescence-activated cell sorting. In (*B*), only the cells from the upper left quadrant (CD25+, CFSE low) are plotted. All cultures were analyzed in triplicate; averages and standard deviations are shown.

3.1.2 Inhibition of Poly(ADP-ribosyl)ation Prevents and Cures *Helicobacter*-induced Gastric Preneoplasia

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Journal: Cancer Research 2010; 70(14) July 15, 2010;
doi:10.1158/0008 5472.CAN-10-0528

Contribution: Planning, performing (except supplemental figure 1) and analysis of experiment; figure assembly, assistance in manuscript writing, revision of the manuscript

Summary: The PARP family of enzymes constitute key players of inflammatory processes and as guardians of genome stability through their ability to post-transcriptionally ADP-ribosylate its targets. We show that the T cell-driven gastric preneoplastic immunopathology that is observed in *Helicobacter*-infected C57BL/6mice is attenuated by systemic administration of PJ34, an NAD^+ analogue and inhibitor of ADP-ribosylation originally designed to inhibit PARP1. The same treatment further efficiently reversed pre-existing lesions, especially when applied in combination with *Helicobacter* eradication therapy. We find that PJ34 exerts its chemopreventive and therapeutic effects by inhibiting the priming and programming of Th-1 polarized T cells in the gut-draining lymph nodes. An essential role for ADP-ribosylation during T-cell activation was further corroborated *in vitro,* as the PAR formation, cytokine secretion, and proliferation induced by CD3/CD28 crosslinking were blocked by PJ34. In conclusion, we propose that targeting ADP-ribosylating enzymes in T cells may represent a novel treatment strategy of gastric premalignant lesions in patients that are refractory to *Helicobacter* eradication therapy.

Cancer Research

Inhibition of ADP Ribosylation Prevents and Cures *Helicobacter*-Induced Gastric Preneoplasia

AU Isabella M. Toller[1], Matthias Altmeyer[2], Esther Kohler[1], Michael O. Hottiger[2], and Anne Müller[1]

Abstract

Q3 Gastric adenocarcinoma develops as a consequence of chronic inflammation of the stomach lining that is caused by persistent infection with the bacterium *Helicobacter pylori*. Gastric carcinogenesis progresses through a sequence of preneoplastic lesions that manifest histologically as atrophic gastritis, intestinal metaplasia, and dysplasia. We show here in several preclinical models of *Helicobacter*-induced atrophic gastritis, epithelial hyperplasia, and metaplasia that the inhibition of ADP ribosylation by the small-molecule inhibitor PJ34 not only prevents the formation of gastric cancer precursor lesions, but also efficiently reverses preexisting lesions. PJ34 exerts its chemopreventive and therapeutic effects by impairing *Helicobacter*-specific T-cell priming and T_H1 polarization in the gut-draining mesenteric lymph nodes. The subsequent infiltration of pathogenic T cells into the gastric mucosa and the ensuing gastric T cell–driven immunopathology are prevented efficiently by PJ34. Our data indicate that PJ34 directly suppresses T-cell effector functions by blocking the IFN-γ production of mesenteric lymph node T cells *ex vivo*. Upon exposure to PJ34, purified T cells failed to synthesize ADP-ribose polymers and to activate the transcription of genes Q2 encoding IFN-γ, interleukin 2, and the interleukin 2 receptor α chain in response to stimuli such as CD3/CD28 cross-linking or phorbol 12-myristate 13-acetate/ionomycin. The immunosuppressive and chemoprotective effects of PJ34 therefore result from impaired T-cell activation and T_H1 polarization, and lead to the protection from preneoplastic gastric immunopathology. In conclusion, ADP-ribosylating enzymes constitute novel targets for the treatment of *Helicobacter*-associated gastric lesions predisposing infected individuals to gastric cancer and may also hold promise for the treatment of other T cell–driven chronic inflammatory conditions and autoimmune pathologies. *Cancer Res; 70(14); OF1–11. ©2010 AACR.*

Introduction

Epidemiologic and experimental studies have established unequivocally that chronic inflammatory diseases predispose to cancers of the liver, cervix, colon, stomach, and several other organs (1). In the stomach, chronic inflammation caused by persistent infection with the Gram-negative bacterial pathogen *Helicobacter pylori* is the most important risk factor for the development of adenocarcinoma (2–5). In a high-risk subset of infected individuals, *H. pylori* causes multifocal corpus-predominant atrophic gastritis, which frequently progresses to intestinal metaplasia, occasionally to dysplasia, and rarely to adenocarcinoma (6, 7). We have shown recently using a C57BL/6 mouse model of *Helicobacter* infection that the early stages of this preneoplastic sequence develop as a result of gastric infiltration by T_H1-polarized, MHC class II–restricted effector T cells (8). This T-cell subset is, on the one hand, absolutely essential for the efficient control of infection and, on the other hand either directly or indirectly induces gastric epithelial transformation (8). Both phenotypes depend on IFN-γ, which is secreted by T_H1-polarized effector T cells both locally at the site of infection and in the gut-draining mesenteric lymph nodes (MLN). Targeting T cells pharmacologically in the infectious process prevents and even reverses established premalignant lesions, confirming that T cells are crucial mediators of gastric preneoplasia (9).

Poly(ADP-ribose) (PAR) is synthesized by the PAR polymerase (PARP) family of enzymes, which use the substrate NAD^+ to ADP-ribosylate acceptor proteins (10–12). PARP enzymes have been implicated in the pathogenesis of several chronic inflammatory and autoimmune conditions, and NAD^+-mimicking inhibitors of poly(ADP-ribosyl)ation efficiently block inflammation in rodent models of Crohn's disease (13) and in allergen-induced airway inflammation models (14), and protect mice from endotoxic shock (15). Since the concept of synthetic lethality of PARP inhibitors in cells with defects in homologous recombination was

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-0528

introduced in 2005 (16, 17), PARP inhibitors have entered the arena of cancer therapy, showing a benefit in breast and ovarian cancer patients with BRCA1- or BRCA2-deficient tumors (18–21).

We show here that the T cell–driven gastric preneoplastic immunopathology observed in *Helicobacter*-infected C57BL/6 mice is attenuated by systemic administration of PJ34, a NAD^+ analogue and inhibitor of ADP ribosylation originally designed to inhibit PARP1 (22). The same treatment further efficiently reversed preexisting lesions, especially when applied in combination with *Helicobacter* eradication therapy. We find that PJ34 exerts its chemopreventive and therapeutic effects by inhibiting the priming and programming of T_H-1 polarized T cells in the gut-draining lymph nodes. An essential role for ADP ribosylation during T-cell activation was further corroborated *in vitro*, as the PAR formation, cytokine secretion, and proliferation induced by CD3/CD28 cross-linking were blocked by PJ34. In conclusion, we propose that ADP-ribosylating enzymes in T cells may represent novel targets for the treatment of gastric premalignant lesions in patients that are refractory to *Helicobacter* eradication therapy.

Materials and Methods

Animal experimentation, *Helicobacter* strains, and pharmacologic treatments

Male C57BL/6, interleukin (IL)-10$^{-/-}$BL/6, CD4$^{-/-}$BL/6, and MyD88$^{-/-}$BL/6 mice (Charles River Laboratories) as well as PARP1$^{-/-}$BL/6 (23) and PARP2$^{-/-}$BL/6 (a kind gift from Q4 Gilbert de Murcia) were maintained in individually ventilated cages and infected at 5 to 6 weeks of age with two consecutive doses of ~10^8 *H. felis* CS1 (ATCC 49179; ref. 24). All animal experiments were approved by the cantonal veterinary office. For *in vivo* inhibition of ADP ribosylation, PJ34 [*N*-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)-*N*,*N*-dimethylacetamide, Enzo Life Sciences] was administered in drinking water supplemented with 0.1 mg/mL Aspartame, resulting in a daily dose of 30 mg/kg of body weight. In cell culture experiments, PJ34 and DAM-TIQ-A [2-Dimethylaminomethyl-4H-thieno (2,3-c)isoquinolin-5-one; Enzo Life Sciences] were each used at 15 μmol/L. Antibiotic eradication therapy was achieved by 2 weeks of daily orogastric treatment with 4.5 mg/mL metronidazole, 10 mg/mL tetracycline hydrochloride (both Sigma-Aldrich), and 1.2 mg/mL bismuth subcitrate (Park-Davis).

Assessment of *H. felis* colonization, gastric IFN-γ production, gastric neutrophil and lymphocytic infiltration, and histopathology

Murine stomachs were dissected longitudinally into equally sized pieces. For the assessment of *H. felis* colonization, total genomic stomach DNA was subjected to quantitative PCR analysis of the *fla*B gene, as previously described (8). The quantitative analysis of gastric IFN-γ expression by real-time reverse transcription-PCR (RT-PCR) and the quantification of neutrophil infiltration by myeloperoxidase activity assay were performed as described (8). Gastric lymphocyte single-cell suspensions were generated by digestion with 0.25 mg/mL collagenase IV (Sigma) and passing through a cell strainer. PARP1 expression was analyzed by immunoblotting of mucosal extract using rabbit anti-PARP1 antibody H-250 (Santa Cruz). Gastric histopathology was graded on Giemsa-stained paraffin sections as described in detail previously (8, 9). All pictures were taken with a Leica Leitz DM RB microscope equipped with a Leica DFC 420C camera. Images were acquired at ×20 magnification using the Leica Application Suite 3.3.0 software. Scale bars indicate 50 μm. The immunomagnetic isolation of lymphocytes from MLN and spleen, and the assessment of lymphocyte proliferation as well as IFN-γ and IL-2 expression by real-time RT-PCR and intracellular staining is described in the Supplementary Methods.

Statistical analysis

P values were calculated using GraphPad prism 5.0 software. The significance of differences in histopathology categories was calculated by Mann-Whitney test, and the significance of numerical differences was calculated by Student's *t* test. All *in vitro* assays were analyzed in triplicate and are shown with SDs.

Results

Inhibition of ADP ribosylation by PJ34 *in vivo* attenuates *Helicobacter*-induced atrophic gastritis

As ADP-ribosylating enzymes have been implicated in chronic inflammatory and autoimmune disorders, and also in the development and progression of certain neoplasms (13, 14, 16, 17), we hypothesized that blocking ADP ribosylation *in vivo* would modulate *Helicobacter*-induced gastric (pre) neoplasia. To validate the use of the NAD^+ analogue PJ34, an inhibitor of PARPs and of ADP ribosylation in general, we first used the standardized human AGS gastric epithelial cell culture model of *H. pylori* infection. PAR formation could be detected in *H. pylori*–infected AGS cells as early as 6 hours postinfection (Supplementary Fig. S1A). SF1 The synthesis of PAR was completely blocked by the addition of PJ34 (Supplementary Fig. S1B), implying that PJ34 readily permeates into cultured cells and efficiently blocks all enzymes involved in *Helicobacter*-dependent PAR formation.

Experimental infection of C57BL/6 mice with the close *H. pylori* relative *H. felis* mimics the human host's response to the bacterium, with gastric lesions manifesting histologically as chronic atrophic gastritis accompanied by epithelial hyperplasia and acidic mucus–positive metaplasia (8, 9, 25, 26). The formation of preneoplastic lesions is strongly accelerated in IL-10$^{-/-}$ mice, which develop atrophy, hyperplasia, and metaplasia as early as 4 weeks postinfection (Fig. 1A) F1 compared with ~12 weeks postinfection in wild-type (WT) mice (8). IL-10$^{-/-}$ mice thus constitute a convenient model for pharmacologic intervention.

IL-10$^{-/-}$ mice were given PJ34 with the drinking water at a daily dose of 30 mg/kg of body weight. The quantitative analysis of gastric histopathology revealed that PJ34 treatment strongly reduced *Helicobacter*-associated inflammation, atrophy, and epithelial hyperplasia and, to a lesser extent, intestinal metaplasia (Fig. 1A). In agreement with the effects of

PJ34 on gastric histopathology, the mucosal infiltration of neutrophils as determined by myeloperoxidase activity assay was reduced in the presence of the inhibitor (Fig. 1B). PJ34 treatment further significantly impaired the clearance of *Helicobacter* that is typically observed in IL-10$^{-/-}$ mice (Fig. 1C). To clarify whether PJ34 exerts its protective effects through inhibition of PARP1, the target for which PJ34 was originally designed, we first analyzed PARP1 expression in the gastric mucosa of infected and uninfected IL-10$^{-/-}$ mice (Fig. 1D). All infected mice showed higher levels of PARP1 than their age-matched uninfected counterparts; PARP1 levels were not influenced by PJ34 (Fig. 1D). To investigate the functional consequences of *Helicobacter*-associated PARP1 accumulation in the gastric mucosa, we crossed IL-10$^{-/-}$ mice with PARP1$^{-/-}$ mice to generate IL-10$^{-/-}$/PARP1$^{-/-}$ animals. We assessed the development of gastritis and preneoplastic lesions in this strain over time compared with the PARP1-proficient parental strain. Remarkably, no significant differences could be observed with respect to the onset or the severity of lesions (5, 14, and 21 d postinfection; Supplementary Fig. S2), implying that PJ34 acts through mechanisms not dependent only on PARP1 to attenuate *Helicobacter*-associated gastric pathology *in vivo*. SF2

Inhibition of ADP ribosylation by PJ34 reverses preexisting lesions

We next examined a possible therapeutic effect of PJ34; to this end, we had to abandon the IL-10$^{-/-}$ model, as these mice clear the infection (Fig. 1C) and spontaneously revert to normal gastric histology by 6 to 8 weeks postinfection

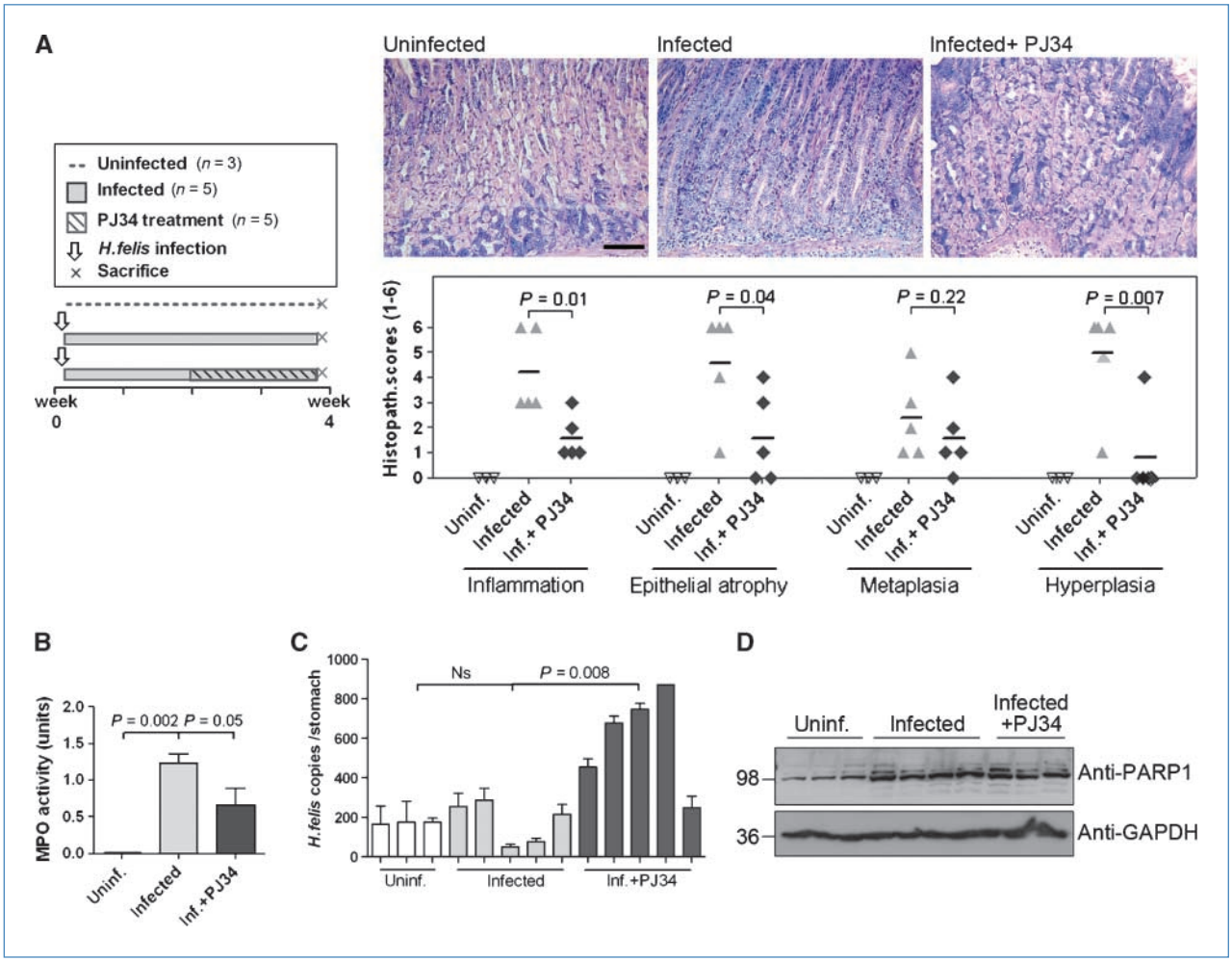

Figure 1. Inhibition of ADP ribosylation prevents *Helicobacter*-induced gastritis and precancerous lesions. IL-10$^{-/-}$ mice were infected for 4 wk with *Helicobacter felis* or remained uninfected. One infected group received PJ34 (30 mg/kg/d) during the last 2 wk of infection. A, a schematic treatment overview, representative Giemsa-stained sections, and histopathology scores are shown for all mice. Gastric histopathology was assessed with respect to chronic inflammation, atrophy, metaplasia, and compensatory epithelial hyperplasia; scores were assigned on a scale of 0 to 6. Horizontal columns, mean values. Scale bars, 50 μm. B, neutrophil infiltration into the stomach was assessed by myeloperoxidase activity assay; columns, means; bars, SD. C, *Helicobacter* colonization was analyzed by quantification of the *flaB* gene copy number by real-time PCR of gastric genomic DNA. Ns, not significant. D, gastric PARP1 expression was analyzed for three to four mice per group by immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels are shown as loading controls. Q8 Q5

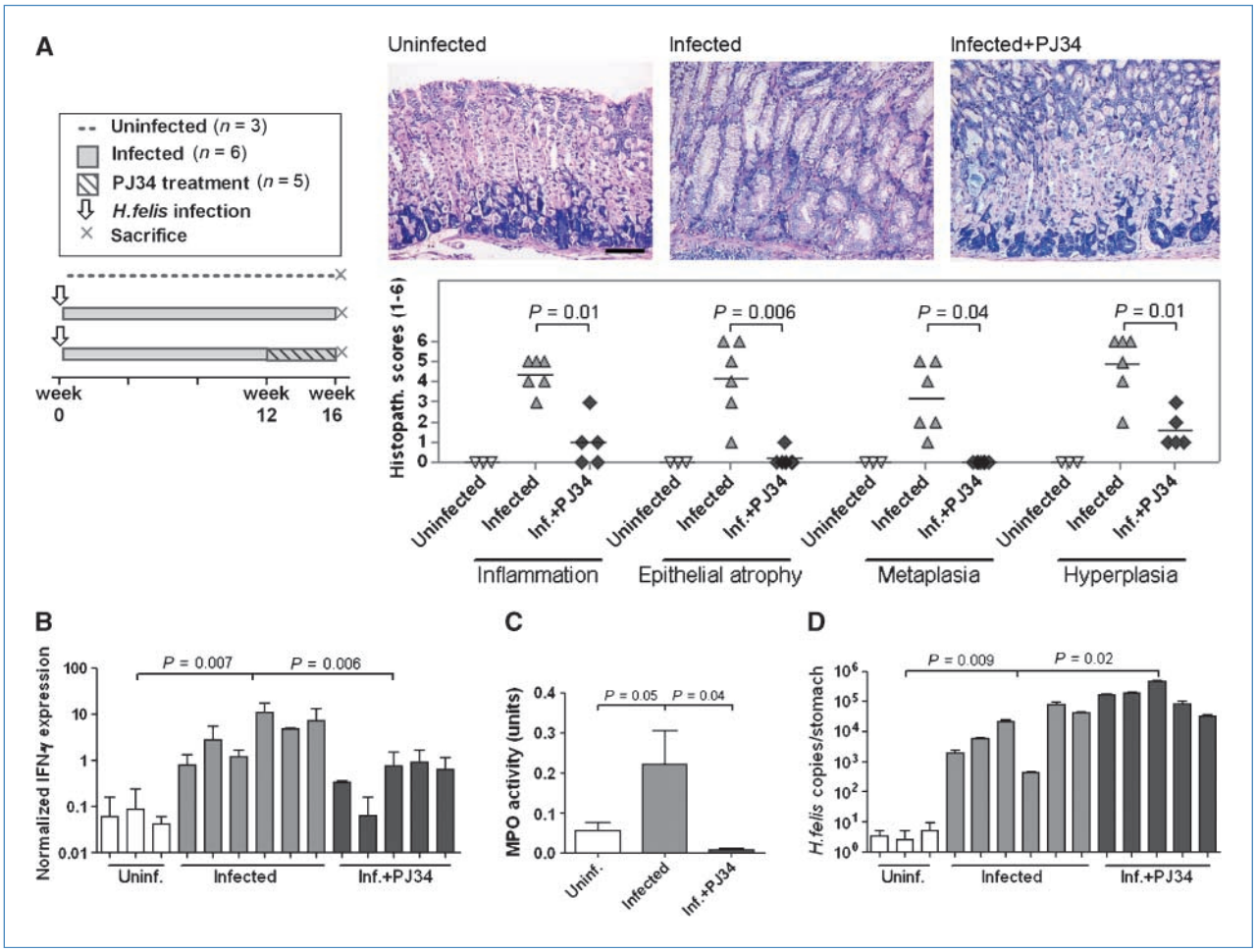

Figure 2. Inhibition of ADP ribosylation reverses preexisting gastritis and precancerous lesions. CD4$^{-/-}$ mice were infected with *H. felis* for 3 mo and either received PJ34 in the 4th month postinfection or remained nontreated. A group of three mice served as uninfected age-matched controls. A, schematic overview, representative Giemsa-stained sections, and histopathology scores. Horizontal columns, mean values. B, IFN-γ transcript levels as quantified by real-time RT-PCR analysis of gastric total RNA. C, neutrophil infiltration as quantified by myeloperoxidase activity assay. D, *H. felis* colonization as analyzed by quantification of the *flaB* gene copy number by real-time PCR of gastric genomic DNA.

(data not shown). For therapeutic studies, we used CD4$^{-/-}$ mice that develop premalignant lesions more consistently than C57BL/6 WT mice by 3 months postinfection, creating a therapeutic window for pharmacologic intervention between 3 and 4 months postinfection (Fig. 2). The increased susceptibility of CD4$^{-/-}$ mice is presumably due to their lack of a CD4$^+$ regulatory T-cell population that could control the immunopathology induced by "pathogenic" effector T cells, which are CD8$^+$ in the absence of CD4$^+$ T cells (27).

CD4$^{-/-}$ mice were infected with *H. felis* for 3 months, and the development of premalignant pathology was confirmed in a small control group (data not shown). The remaining mice either received PJ34 in the 4th month postinfection or remained nontreated. The treatment with PJ34 efficiently reduced all parameters of gastric histopathology (Fig. 2A). The regression of lesions was accompanied by a significant reduction of gastric IFN-γ production and neutrophil infiltration (Fig. 2B and C). In line with our previous observations (8, 9), the reduction of IFN-γ and mucosal neutrophils was associated with an increase in *Helicobacter* colonization (Fig. 2D), which is indicative of a reversal to normal gastric mucosal architecture and physiology.

The regression of preneoplastic lesions is not sustained under conditions of ongoing *Helicobacter* colonization

To confirm these results in an independent model, we took advantage of a mouse strain that lacks the gene encoding the adaptor protein MyD88. MyD88$^{-/-}$ mice develop severe preneoplastic lesions as early as 1 month postinfection (Supplementary Fig. S3A), presumably due to a defect in immune counter-regulation.[3] However, in contrast to CD4$^{-/-}$ and IL-10$^{-/-}$ mice, which efficiently reduce *H. felis* colonization or even clear the infection, respectively (Figs. 1 and 2), MyD88$^{-/-}$

[3] A. Sayi, I. Toller, A. Mueller, unpublished observations.

mice are colonized heavily for at least 3 months postinfection (Fig. 3). We used the therapeutic window between 1 and 3 months postinfection to test whether the reversal of preneoplastic lesions by PJ34 treatment would be sustained after termination of the treatment, despite ongoing *H. felis* colonization, or would require the concomitant eradication of the infection (Fig. 3).

MyD88$^{-/-}$ mice were infected for 1 month, and the development of pathology was verified in a small control group (Supplementary Fig. S3A and D). The remaining mice either received PJ34 for the subsequent month, were subjected to eradication therapy in weeks 5 and 6 postinfection, or received both treatments. At 2 months postinfection, the reversal of preexisting lesions and the efficiency of eradication therapy were verified in additional control groups (Supplementary Fig. S3B and D). All remaining mice were maintained for a 3rd month and then analyzed with respect to their colonization and histopathology. Mice that had been continuously colonized throughout the 3-month time course had all developed severe pathology (Fig. 3A). The regression

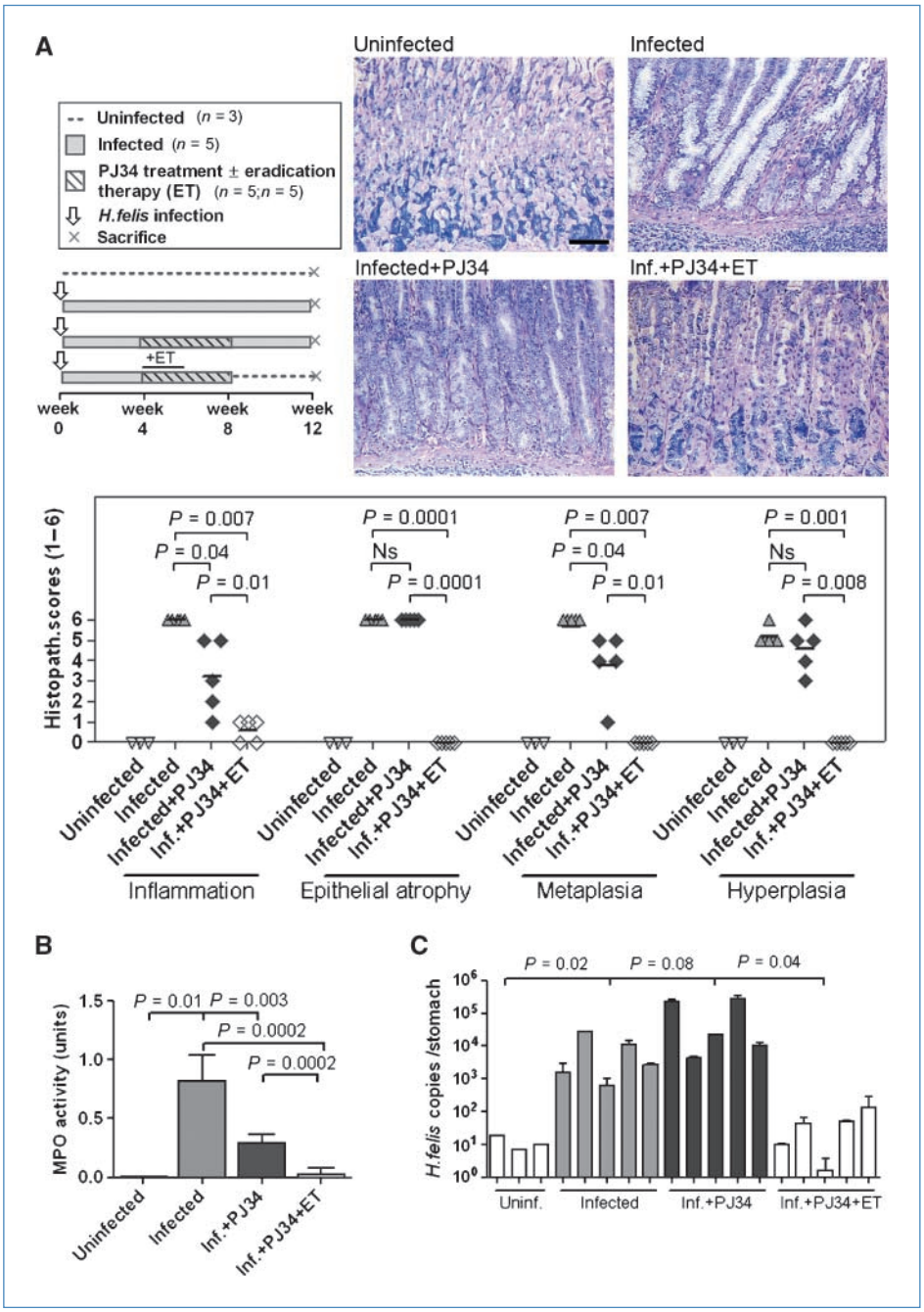

Figure 3. The regression of preneoplastic lesions is stable under conditions of simultaneous PJ34 treatment and *Helicobacter* eradication. MyD88$^{-/-}$ mice were infected for 3 mo and received PJ34 in the 2nd month with or without eradication therapy in weeks 5 and 6 postinfection. Additional mice served as infected or uninfected age-matched controls. Control groups were sacrificed after 1, 2, and 3 mo to verify the success of the respective treatments (see Supplementary Fig. S3). A, schematic overview, representative Giemsa-stained sections, and histopathology scores; horizontal columns, means. B, neutrophil infiltration into the gastric mucosa as quantified by myeloperoxidase assay. C, *H. felis* colonization as quantified by real-time PCR of the *flaB* gene.

of preneoplastic lesions induced by PJ34 treatment during the second month was transient, as the mice relapsed once the treatment was discontinued (Fig. 3A). In contrast, mice that had been cured of their infection while receiving PJ34 showed sustained regression of their lesions, i.e., the effect of the treatment was stable in this group (Fig. 3A). As reported previously (28), eradication therapy by itself was also effective at reversing existing lesions (Supplementary Fig. S3C and D), indicating that *Helicobacter* eradication and the inhibition of ADP ribosylation have comparable effects in mice. In contrast, eradication therapy typically fails to cure the human counterpart lesions (29), suggesting that the consecutive treatment with antibiotics and ADP ribosylation inhibitors would have a beneficial effect over eradication therapy alone in humans.

The effects on gastric histopathology were reflected in the levels of neutrophil infiltration, which decreased more dramatically in the group that was subjected to both PJ34 treatment and eradication therapy than in the group that received PJ34 only (Fig. 3B). *Helicobacter* levels were not significantly different between the two infected groups with or without PJ34 treatment; only background levels of *Helicobacter*-specific PCR products were detectable upon eradication therapy (Fig. 3C). In conclusion, the pharmacologic inhibition of ADP ribosylation for therapeutic purposes is temporarily successful in reversing preexisting lesions, but is sustained only if the underlying infection is eradicated.

Inhibition of ADP ribosylation prevents T-cell priming in MLNs

We have reported previously that gastric IFN-γ responses to *Helicobacter* infection correlate with the level of gastric T-cell infiltration and preneoplastic pathology (8, 9). This association was again confirmed in this study (Figs. 1 and 2). The proportion of T cells producing IFN-γ (i.e., T_H-1–polarized effector T cells) in the MLN is a reliable indicator of *Helicobacter*-specific T-cell responses and a good predictor of gastric pathology (9). To assess whether inhibition of ADP ribosylation modulates gastric histopathology by interfering with T-cell priming, we infected 10 WT C57BL/6 mice with *H. felis* and treated one half of the group with PJ34 for 1 month. The rest remained nontreated; additional mice served as uninfected, nontreated controls. IFN-γ–positive T cells were quantified in single-cell MLN preparations of individual mice by intracellular staining for IFN-γ combined with surface staining for CD4 (Fig. 4A). The proportion of IFN-γ^+ cells increased in the $CD4^+$ and $CD4^-$ fractions as a result of the infection; this increase was suppressed by PJ34 treatment (Fig. 4A). As a consequence of impaired T-cell priming in the MLN due to PJ34 treatment, fewer leukocytes were present in single-cell preparations of the corresponding stomachs (Fig. 4B, top), and fewer of these gastric leukocytes stained positive for CD4 (Fig. 4B, bottom). To test whether the inhibition of T_H-1 polarization was due to a direct effect of PJ34 on IFN-γ production, we cultured single-cell preparations from five additional *H. felis*–infected WT mice for 3 days in the presence or absence of PJ34. All treated cultures revealed a reduction of IFN-γ expression compared with the respective controls (Fig. 4C); the simultaneous visualization of PAR showed that PJ34 indeed significantly reduced PAR formation under these circumstances (Fig. 4C). Finally, we pulsed immunomagnetically isolated MLN-derived dendritic cells with *Helicobacter* sonicate and cocultured them with splenic $CD4^+$ T cells isolated from uninfected or infected donors (Fig. 4D); the infection-dependent IFN-γ response of the T cells was significantly reduced by PJ34 treatment, as were the background levels of T-cell activation (Fig. 4D). In conclusion, the T-cell priming, T_H-1 polarization, and IFN-γ production that are a prerequisite for the development of gastric *Helicobacter*-associated pathology are blocked by PJ34, suggesting that T cells represent the key target of PJ34 in this scenario.

T-cell activation is impaired by inhibition of ADP ribosylation

T-cell activation requires two external signals, an antigen-specific signal that is transmitted through the TCR/CD3 complex and a costimulatory signal received by activation of CD28 (30). Primary T cells are induced to proliferate, to express the activation marker CD25, and to produce and secrete IFN-γ upon CD3/CD28 cross-linking or treatment with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Fig. 5). We isolated splenic $CD4^+CD25^-$ T cells and stimulated them with anti-CD3/anti-CD28 monoclonal antibody–coated beads or PMA/ionomycin in the presence or absence of either PJ34 or an alternative inhibitor of ADP ribosylation, DAM-TIQ-A. Both treatments induced proliferation of the T cells as assessed by carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution, and proliferation was blocked by both compounds (Fig. 5A). PJ34 was more active than DAM-TIQ-A at the same final concentration. The concomitant upregulation of CD25 was reduced as well (data not shown; Fig. 6B), as was IFN-γ production (Fig. 5C). We conclude that inhibition of ADP ribosylation by either PJ34 or DAM-TIQ-A significantly blocks $CD4^+CD25^-$ T-cell activation triggered by two alternative stimuli.

To investigate whether the effect of blocking ADP ribosylation was attributable to either PARP1 or PARP2, we stimulated parallel preparations of immunomagnetically isolated WT, $PARP1^{-/-}$, and $PARP2^{-/-}$ $CD4^+CD25^-$ T cells by CD3/CD28 cross-linking in the absence or presence of PJ34 and quantified their proliferation (Supplementary Fig. S4). T cells of all three genotypes responded equally well to CD3/CD28 cross-linking; no differences were detected with respect to proliferation (Supplementary Fig. S4), CD25 upregulation, or IFN-γ production (data not shown). Furthermore, we found that PJ34 had similarly suppressive effects on all three genotypes (Supplementary Fig. S4), suggesting that neither PARP1 nor PARP2 are the sole targets of PJ34 in T cells.

To test whether T-cell inhibition by PJ34 is restricted to $CD4^+$ T cells or is a common feature shared by other T-cell subsets, we immunomagnetically isolated splenic $CD8^+$ T cells, subjected them to CD3/CD28 cross-linking, and measured their proliferation (Supplementary Fig. S5). PJ34 treatment indeed blocked the proliferation of $CD8^+$ T cells as efficiently as that of $CD4^+$ T cells, implying that PJ34 has

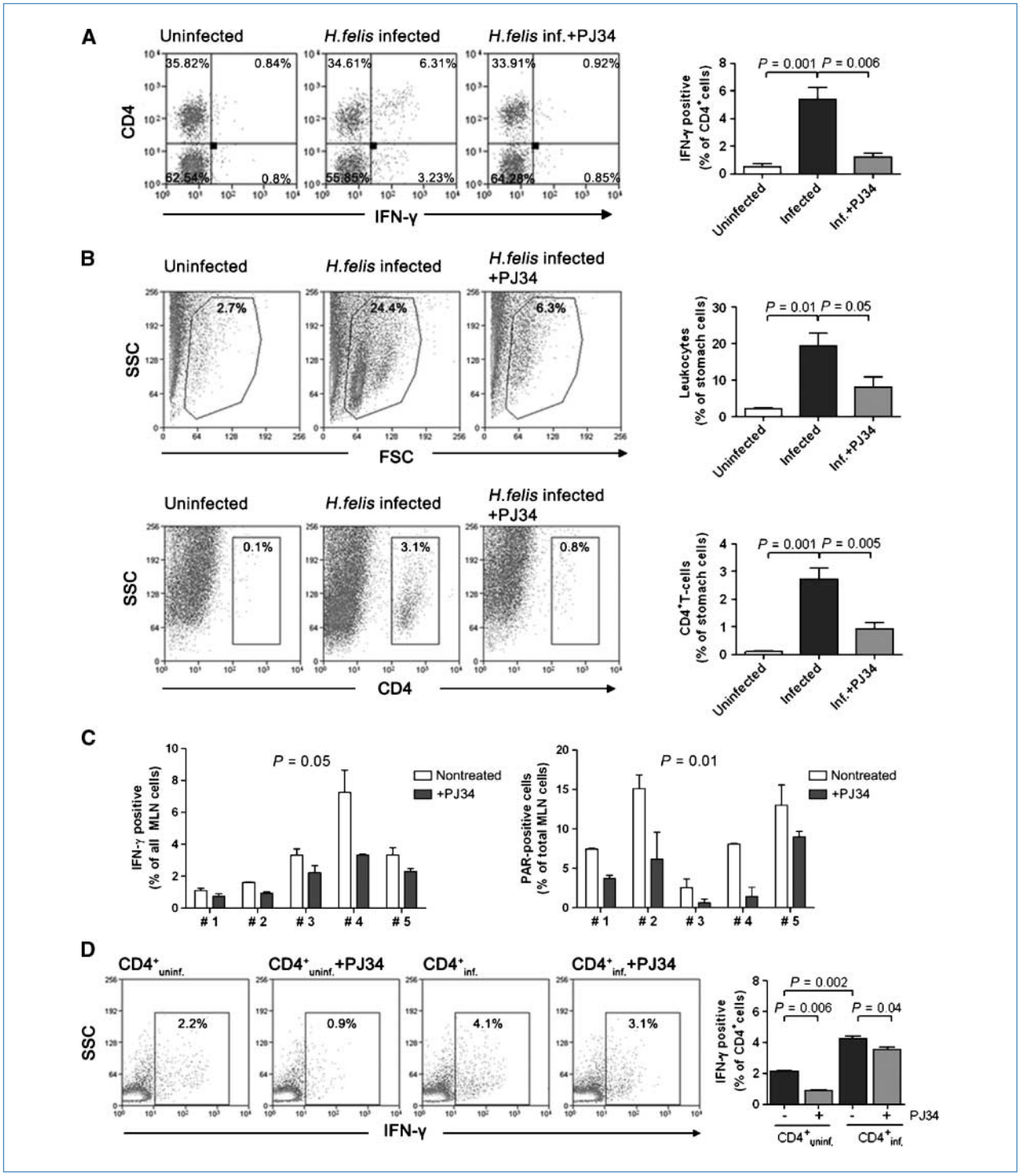

Figure 4. Inhibition of ADP ribosylation impairs T-cell priming and T_H-1 differentiation in the MLNs. A and B, C57BL/6 mice were infected for 1 mo with *H. felis* or remained uninfected; of the infected mice, a subset received PJ34 during the entire course of infection. Single-cell suspensions were generated from the MLN (A) and stomachs (B) of all five mice per group and analyzed individually by flow cytometry. Representative dot plots of CD4 and intracellular IFN-γ signals and averages of all MLN cultures are shown (A). Similarly, representative dot plots and group averages are shown for the percentage of leukocytes and $CD4^+$ T cells per total stomach cells (B, top and bottom). C, additional MLN cultures from five infected mice (numbered 1–5) were cultured for 3 d in the presence or absence of 15 μmol/L PJ34 before intracellular staining for IFN-γ and ADP-ribose polymers. D, immunomagnetically isolated MLN-derived dendritic cells from uninfected donors were pulsed with *Helicobacter* sonicate for 16 h and cocultured with splenic $CD4^+$ T cells from uninfected or infected donors before staining of CD4 and intracellular IFN-γ. All events in the $CD4^+$ gate are plotted; representative dot plots and group averages are shown. *P* values were calculated by unpaired (A, B, and D) and paired (C) Student's *t* test.

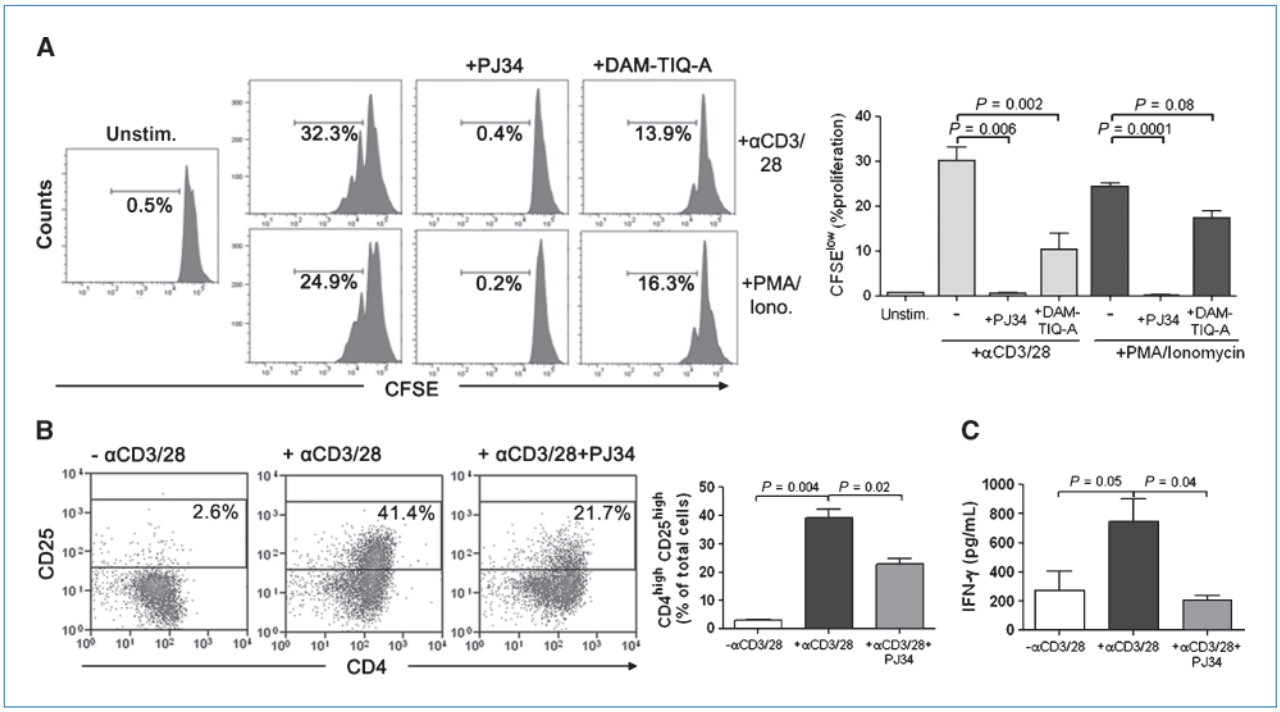

Figure 5. Inhibition of ADP ribosylation prevents T-cell activation by CD3/CD28 cross-linking or treatment with PMA/ionomycin. $CD4^+CD25^-$ cells were isolated from single cell preparations of the combined MLN of five mice. The cells were labeled with CFSE and stimulated for 3 d with αCD3/28-coated beads or PMA/ionomycin; 15 μmol/L PJ34 or 15 μmol/L DAM-TIQ-A was added where indicated. A, CFSE dilution as a measure of proliferation was assessed flow cytometrically. Representative histograms (left) as well as the averages of triplicate cultures (right) are shown. B, CD25 expression was assessed by surface staining. Representative dot plots and averages of triplicate cultures are shown. C, the αCD3/CD28-induced secretion of IFN-γ into the culture supernatants was measured by ELISA for the same cultures as shown in B.

broad T-cell immunosuppressive capacity. Interestingly, splenic $CD4^+$ T cells isolated from PJ34-treated mice at the time of sacrifice were as responsive to CD3/CD28 cross-linking–induced activation as cells from nontreated donors (data not shown), suggesting that the effects of PJ34 *in vivo* are transient and do not permanently silence this important branch of the immune system. In conclusion, PJ34 acts as an efficient T-cell immunosuppressant *in vitro*, suggesting that it may target T cells in a similar manner also *in vivo*.

Inhibition of ADP ribosylation blocks *il-2* and *ifng* gene transcription

Activated T cells require IL-2 for growth (31); therefore, some of the earliest markers of T-cell activation are the up-regulation of the high-affinity α-chain of the IL-2 receptor (CD25; Fig. 5B) and the production of IL-2. IL-2 acts on the producing T cell and its neighbors to sustain their proliferation in an auto and paracrine manner (31). To monitor PAR formation during early stages of T-cell activation upon CD3/CD28 cross-linking, we stained intracellular ADP-ribose polymers after 2, 6, and 16 hours with an antibody that recognizes polymers >16 units in length. PAR formation gradually increased in this time frame and was blocked efficiently by PJ34 (Fig. 6A). Transcription of the *il-2* and *ifng* genes was detectable as early as 2 hours after CD3/CD28 cross-linking (Fig. 6B and C) and remained high after 6 and 16 hours (data not shown). The induction of *il-2* and *ifng* transcripts could be prevented by PJ34, but was independent of PARP1 and PARP2 (Fig. 6B and C), suggesting that the two PARP family members are redundant or that additional PJ34-sensitive ADP-ribosylating enzymes function in the transcriptional activation of T cells. The results indicate that PJ34 blocks T-cell activity by interfering with the autocrine, IL-2–driven positive feedback loop required for sustained T-cell proliferation, and suggest that ADP ribosylation is required for the initiation of the transcriptional program activating T-cell effector functions.

Discussion

Inhibitors of ADP-ribosylating enzymes have attracted attention lately as new compounds for cancer therapy (16, 17, 19, 20), and for the treatment of chronic inflammatory diseases and autoimmune pathologies (13–15, 32). We show here that a broad range inhibitor of ADP ribosylation, PJ34, is capable of preventing and also of curing the *Helicobacter*-associated, T cell–driven immunopathology that precedes gastric cancer development. Several pieces of evidence indicate that pathogenic T cells, and not *Helicobacter*-infected epithelial cells (which also upregulate PAR in the infectious process), are the key targets of PJ34 inhibition in our preclinical model: (*a*) The gastric expression of IFN-γ, which is produced

exclusively by infiltrating $CD4^+$ T cells, is strongly reduced *in vivo*; (*b*) the priming and polarization of T_H1 T cells in the gut-draining lymph nodes is impaired both *in vivo* and *ex vivo* in cocultures of T cells with *Helicobacter* antigen–pulsed MLN-derived dendritic cells; (*c*) IFN-γ secretion by explanted MLN cells from infected mice cultured *ex vivo* is blocked, and (*d*) these effects are presumably due to the PJ34 sensitivity of *il-2* gene transcription as shown in an *ex vivo* model of T-cell stimulation.

ADP-ribose polymers are detectable in T cells only hours after their activation by CD3/CD28 cross-linking; the time frame of PAR formation suggests a role for PAR in the transcriptional activation of genes encoding IL-2, IFN-γ, and the IL-2 receptor α-chain. In line with this observation, the inhibition of ADP ribosylation in T cells by PJ34 blocks *il-2* and *ifng* gene transcription and, presumably as a consequence of these early effects, prevents T-cell proliferation in response to CD3/CD28 cross-linking– and PMA/ionomycin-induced activation. Our *ex vivo* results suggest that PARP1, PARP2, and possibly other PJ34-sensitive PARP family members have redundant roles during T-cell activation. Our finding that IL-$10^{-/-}$/$PARP1^{-/-}$ mice develop gastritis and epithelial pathology with similar kinetics as the parental IL-$10^{-/-}$ $PARP1^+$ strain argues in favor of a redundant role of PARP enzymes also *in vivo*. We conclude from the combined results that ADP ribosylation by PJ34-sensitive enzymes is an essential step in the activation and/or T_H1 polarization of pathogenic T cells, which directly cause the gastric pathology preceding gastric neoplasia.

The concept of treating T cell–driven diseases such as chronic inflammatory disorders or autoimmune diseases with inhibitors of ADP-ribosylating enzymes is not new. PARP inhibitors have for instance been successfully used in preclinical models of asthma and colitis (13, 14, 33–35). PARP inhibition and PARP1 gene deletion were shown to protect mice from ovalbumin-induced allergic airway inflammation (14). In rodent models of Crohn's disease–like colitis, the inhibition of ADP ribosylation was sufficient to prevent disease (13, 33–35). Protection was accompanied by decreased levels of inflammatory and T cell–derived cytokines

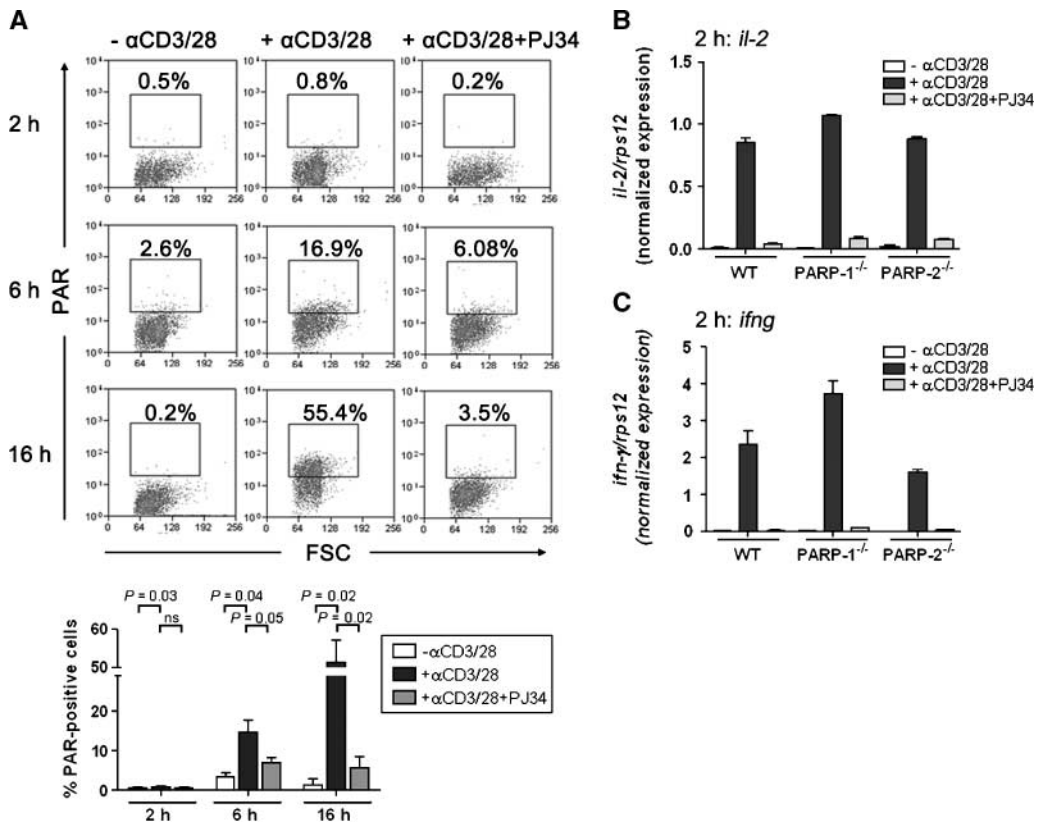

Figure 6. CD3/CD28 cross-linking–induced PAR formation and *il-2* and *ifng* gene transcription are blocked by PJ34. A, PAR formation in $CD4^+CD25^-$ T cells upon CD3/CD28 cross-linking was analyzed by intracellular staining; cells were stimulated with anti-CD3/CD28–coated beads in the presence or absence of 15 μmol/L PJ34 for 2, 6, and 16 h as indicated. Representative dot plots (top) as well as the averages of triplicate cultures (bottom) are shown. B and C, parallel cultures treated for 2 h as described in A were subjected to RNA isolation and quantitative RT-PCR analysis of *il-2* (B) and *ifng* (C) gene expression. Parallel $CD4^+CD25^-$ T-cell cultures from $PARP1^{-/-}$ and $PARP2^{-/-}$ mice were included as well. Transcript levels of *il-2* and *ifng* were normalized to *rps12* levels. Columns, mean; bars, SD.

and a restoration of colonic barrier functions (13, 33–35). Overall, substantial preclinical evidence is available to support a role for ADP ribosylation also in other T cell–driven immune and autoimmune pathologies, suggesting a more general mechanism and a broad applicability of ADP ribosylation inhibitors to these indications.

One of the least anticipated findings of our study was the almost complete reversion of preexisting *Helicobacter*-induced gastric lesions by PJ34 treatment. This result indicates that inhibitors of ADP ribosylation, for which safety data are now available due to their extensive testing in trials of metastatic breast and other cancers (19, 20), might be useful compounds for the treatment of symptomatic *H. pylori*–infected patients. Most patients presenting with atrophic gastritis, metaplasia, or dysplasia are routinely subjected to eradication therapy targeting the underlying infection; however, eradication is only partly efficient in reversing atrophy and fails in the treatment of metaplasia and dysplasia (29). As patients with any of these conditions have an at least 10-fold increased risk of developing gastric cancer compared with histologically unapparent infected individuals, and are currently subjected to an unsatisfactory watch-and-wait strategy, there is a clear unmet need for new treatment options for this patient group. We conclude based on the results obtained by our combined treatment with PJ34 and antibiotics that this inhibitor, or other compounds mimicking NAD^+, may successfully reverse gastric preneoplastic lesions in humans if administered together with or after eradication therapy. In summary, we provide evidence that ADP ribosylation is required for T-cell activation, and that inhibition of ADP-ribosylating enzymes efficiently impairs the T_H-1–polarized T-effector cell responses and the associated gastric immunopathology that are a hallmark of chronic *Helicobacter* infection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. Q7

Acknowledgments

We thank Dirk Bumann, Christoph Dehio, Josef Jiricny, Isabelle Arnold, Iris Hitzler, and Ayca Sayi for helpful discussions.

Grant Support

Swiss National Science foundation grants 310030-127589 and 3100A0-113452 (A. Müller), and 31-109315.05 and 31-122421 (M. Altmeyer). Additional funding was supplied by the University Research Priority Program in Systems Biology/Functional Genomics, the UBS foundation, the Swiss Cancer League and the Nils and Desiree Yde foundation (A. Müller), and by the Kanton of Zurich (M.O. Hottiger).

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Received 02/13/2010; revised 04/27/2010; accepted 05/03/2010.

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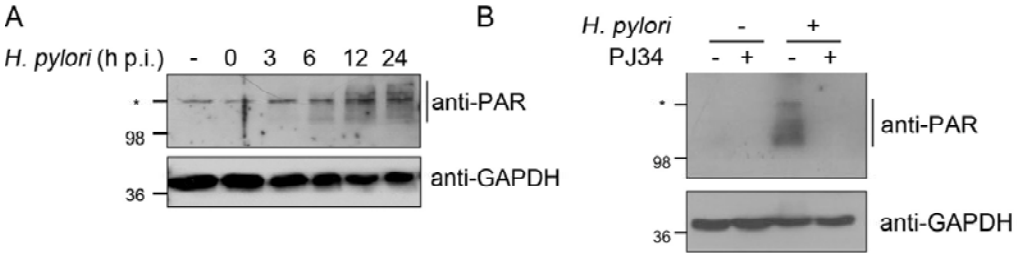
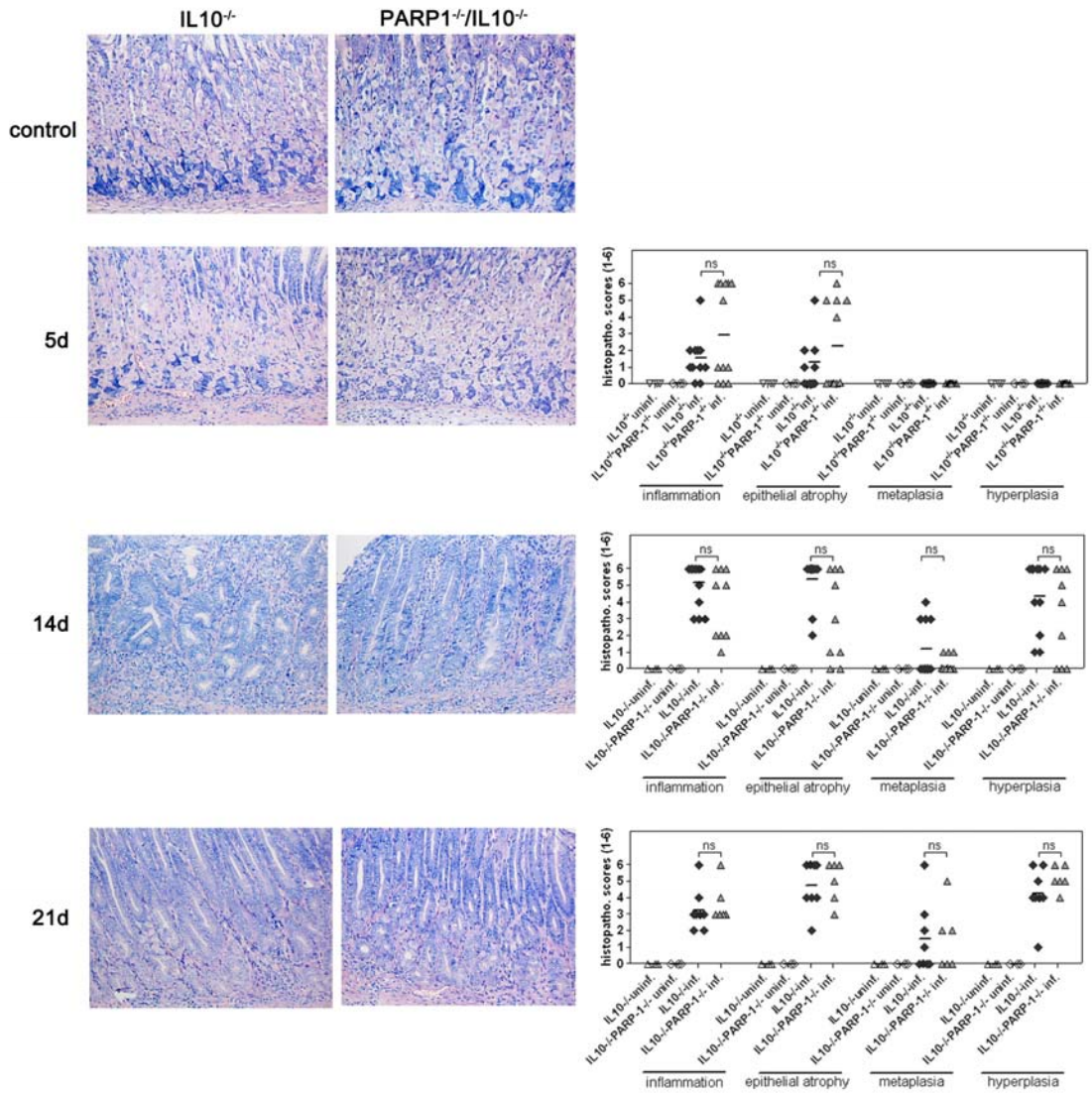
1. Supplemental Figures:

Supplemental Figure 1



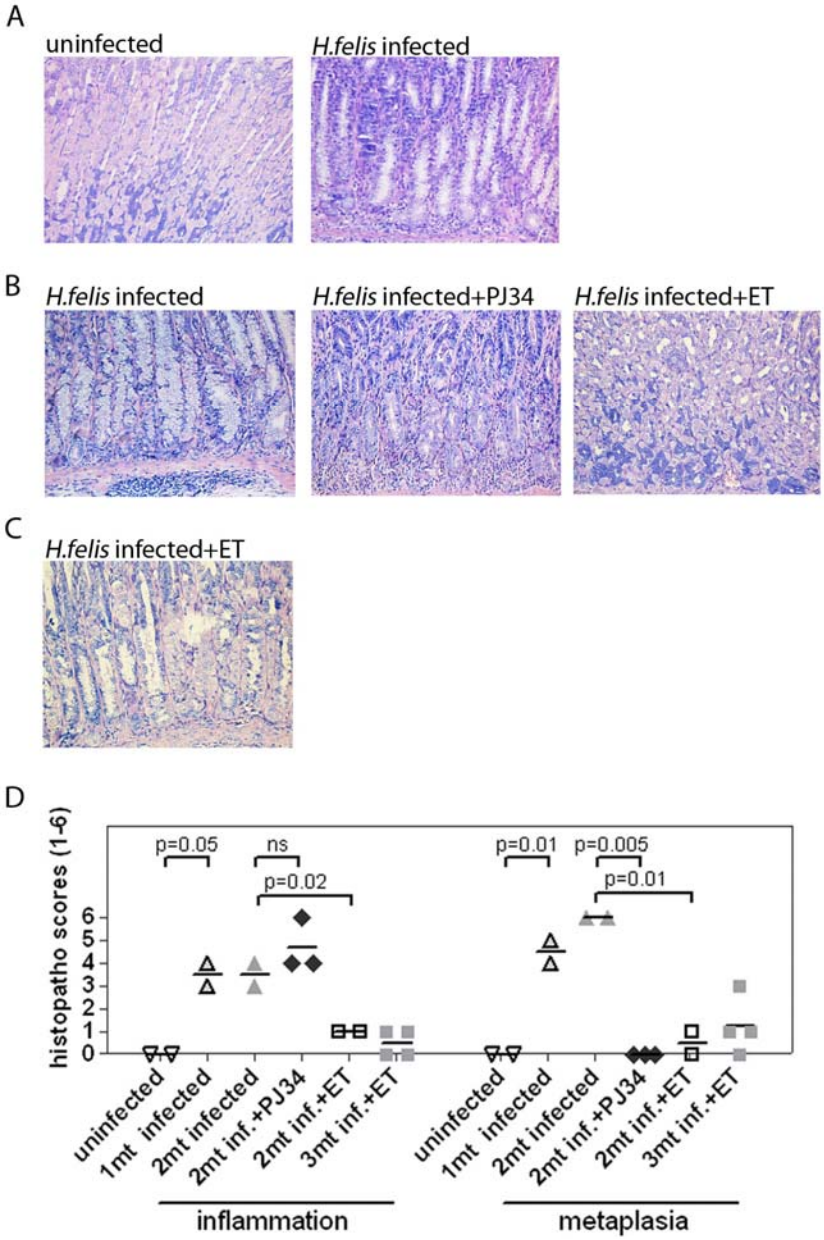
Supplemental Figure 1: The NAD$^+$ analog PJ34 prevents *H. pylori*-induced PAR synthesis in gastric epithelial cells.
(**A**) Human gastric epithelial cells (AGS; purchased from ATCC, CRL 1739) were infected with *H. pylori* strain G27 for 0, 3, 6, 12 and 24 hours. PAR formation was detected by immunoblotting. (**B**) AGS cells were infected with *H. pylori* for 24 hours in the presence or absence of 15μM PJ34. PAR formation was analyzed by immunoblotting.

Supplemental Figure 2



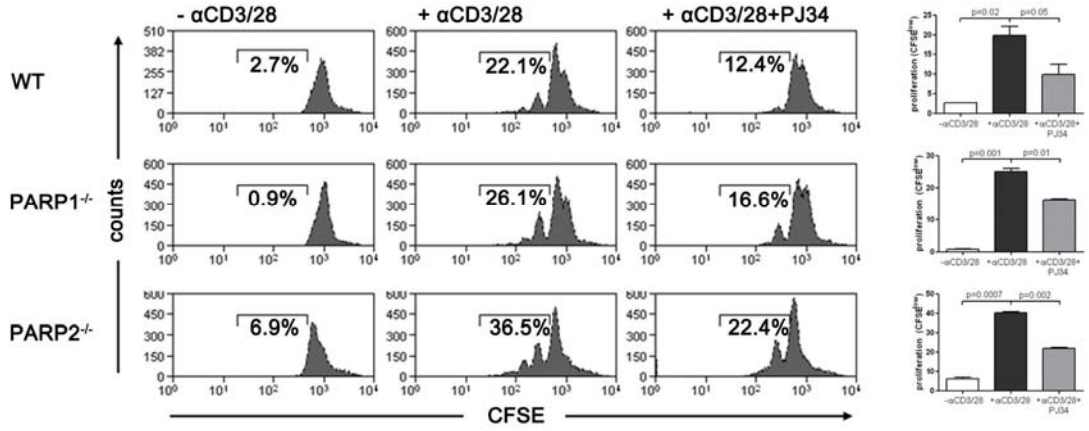
Supplemental Figure 2: PARP1 does not contribute to *H. felis*-induced gastric pathology *in vivo*. IL10$^{-/-}$ and IL10$^{-/-}$/PARP1$^{-/-}$ mice were infected with *H. felis* for 5, 14 and 21 days. Gastric histopathology was assessed with respect to chronic inflammation, atrophy, metaplasia and compensatory epithelial hyperplasia; scores were assigned to every mouse on a scale ranging from 0-6. The horizontal bars represent mean values. Representative Giemsa-stained sections are shown in the left panels.

Supplemental Figure 3



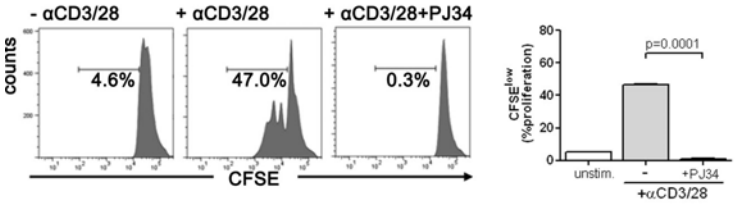
Supplemental Figure 3: Gastric histopathology of *H. felis*-infected MyD88$^{-/-}$ mice that have received PJ34 treatment or eradication therapy. (**A**) Two Myd88$^{-/-}$ mice were infected for one month and compared to two age-matched uninfected controls with respect to gastric histopathology. (**B**) Seven additional infected mice were analyzed two months p.i.; of these, three were treated with PJ34 (30mg/kg per day) for the second month of infection, whereas two remained untreated and two more received eradication therapy during weeks 5 and 6 p.i. (**C**) Four additional mice were analyzed three months p.i.; all four had received eradication therapy in weeks 5 and 6 p.i., but had not received PJ34. Giemsa-stained sections of representative animals are shown in A-C. (**D**) Gastric histopathology scores for all mice included in A-C.

Supplemental Figure 4



Supplemental Figure 4: T-cell proliferation upon CD3/CD28 crosslinking does not depend on PARP1 or PARP2.
$CD4^+CD25^-$ cells were immunomagnetically isolated from WT, $PARP1^{-/-}$ and $PARP2^{-/-}$ spleens. The cells were labeled with CFSE and stimulated with αCD3/28 coated beads for three days; 15μM PJ34 was added where indicated. The CFSE dilution was assessed flow-cytometrically; numbers indicate the percentages of $CFSE^{low}$ (proliferating) cells. Representative histograms as well as the averages of triplicate cultures are shown.

Supplemental Figure 5



Supplemental Figure 5: CD8$^+$ T-cell proliferation upon CD3/CD28 crosslinking is blocked by PJ34. CD8$^+$ T-cells were immunomagnetically isolated from the combined MLN of five infected animals, labeled with CFSE and stimulated with αCD3/28 coated beads; 15μM PJ34 was added where indicated. CFSE dilution was quantified as a measure of proliferation. Representative histograms as well as the averages of triplicate cultures are shown.

2. Supplemental Methods:

Lymphocyte proliferation assays and flow cytometry

CD4$^+$CD25$^-$ and CD8$^+$ T-cells were immunomagnetically purified from single cell suspensions of spleens, mesenteric lymph nodes or stomach tissue (R&D Systems, Minneapolis, USA). Cells were labelled with 5μM carboxyfluorescein succinimidyl ester (CFSE) and stimulated with either anti-CD3/CD28-coated beads (Invitrogen) or PMA (50ng/ml) and ionomycin (0.5mg/ml) (both from Sigma). The CFSE dilution was assessed flow cytometrically. IFN-γ secretion was measured by ELISA (BD Biosciences, San Diego, CA, USA). IL-2 and IFN-γ expression was determined by real time RT-PCR using primers specified previously for IFN-γ (9) and using the following primers for IL-2: 5'-GCGCACCCACTTCAAGCT -3'; reverse: 5'-CCTGCTTACAACACATAAGGC-3'. For the immunomagnetic isolation of dendritic cells, single cell MLN preparations were labeled with a biotinylated CD11c-specific antibody (clone HL3, BD Biosciences) followed by magnetic isolation with streptavidin-coated beads (R&D Systems). DC were pulsed for 16 h with 10μg/ml *Helicobacter* sonicate prior to co-culturing for 4 days with CD4$^+$ T-cells. For staining of intracellular IFN-γ, cultures were stained for CD4 (clone RM4-5, eBioscience, San Diego, USA) and/or CD25 (clone PC-61.5, R&D Systems) prior to fixation, permabilization and IFN-staining. XMG1.2, BD Biosciences). Poly(ADP-ribose) was detected using a polyclonal anti-PAR antibody (no. 551813, BD Biosciences). FACS analyses were performed on a Cyan9 instrument (Beckman Coulter).

3.2 Unpublished Articles

3.2.1 *Helicobacter pylori* causes potentially genotoxic DNA double strand breaks in infected epithelial and U2OS cells

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Contribution: Planning, performing and analysis of experiment, figure assembly, assistance in manuscript writing.

Summary: *Helicobacter*-associated chronic inflammation is a well known risk factor for gastric cancer development due to genotoxic byproducts secreted by inflammatory cells, e.g. reactive oxygen species by neutrophils and IFN-γ by immune cells and subsequent acquisition of tumor-promoting mutations. Helicobacter-induced carcinogenesis occurs in sequence of precancerous lesions which manifest histologically as atrophic gastritis, intestinal metaplasia and dysplasia. During this process, various genetic aberrations occur including mutations in *TP53* and other guardians of the genome. In this study we report that *Helicobacter* infection of various cell lines leads to DNA double strand breaks (DSB) and DNA damage dependent signalling represented by 53BP1 and MDC1 foci formation. This process occurs in an infection dose-dependent manner, requires cell contact with live bacteria and is largely independent of Helicobacter-induced reactive oxygen and the *H. pylori* virulence determinants VacA and the Cag pathogenicity island (CagPAI). We further show, that Helicobacter-associated DSB induction is reversible but requires prior bacterial killing. In conclusion, we provide evidence that *Helicobacter* infection directly contributes to gastric carcinogenesis by harming the host cellular DNA, providing a mechanistic explanation for the carcinogenic properties of this bacterial pathogen.

Helicobacter pylori infection causes potentially genotoxic DNA double strand breaks and triggers DNA damage signalling in eukaryotic host cells

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Abstract

Helicobacter-associated chronic inflammation is a well known risk factor for gastric cancer development. *Helicobacter*-induced carcinogenesis occurs in a sequence of precancerous lesions which manifest histologically as atrophic gastritis, intestinal metaplasia and dysplasia. During this process, various genetic aberrations occur including mutations in *TP53*, E-cadherin and the *p16* tumor suppressor genes that support tumor development. In this study we report that *Helicobacter* infection of various cell lines and primary gastric epithelial cells leads to DNA double strand breaks (DSB) and activation of DNA damage signalling pathways indicated by 53BP1 and MDC1 foci formation. This process occurs in an infection dose-dependent manner, requires cell contact with live bacteria and is largely independent of *Helicobacter*-induced reactive oxygen species and the *H. pylori* virulence determinants VacA and the Cag pathogenicity island. We further show that *Helicobacter*-associated DSB induction is reversible upon bacterial elimination. In conclusion, we provide evidence that *Helicobacter* infection directly contributes to gastric carcinogenesis by damaging the host DNA, providing an explanation for the carcinogenic properties of this bacterial pathogen.

Introduction

Chronic infection with the human bacterial pathogen *Helicobacter pylori* causes gastritis and peptic ulceration[1] and increases the carriers' risk of developing gastric cancer[2, 3] or gastric mucosa-associated lymphoid tissue (MALT) lymphoma.[4, 5] The epidemiological association between *H. pylori* infection and gastric adenocarcinoma has been confirmed experimentally in rodent models using Mongolian gerbils[6] and C57BL/6 mice.[7-10] Both epidemiological and experimental data suggest that bacterial virulence determinants, host genetic factors and environmental influences determine whether or not *Helicobacter*-induced gastritis will progress to gastric cancer.[9, 11] In particular, *H. pylori*'s Cag pathogenicity island, certain pro-inflammatory cytokine promoter polymorphisms and a high salt diet have been

identified as co-factors affecting gastric cancer risk.[2, 6, 7, 12] The Lauren classification describes two main histological types of gastric cancer,[13] both of which have been linked to *H. pylori* infection.[3, 14] The diffuse type is often diagnosed in younger patients, has a hereditary component and arises directly in the chronically inflamed stomach without the involvement of histologically defined precursor lesions.[15] In contrast, the intestinal type mostly affects older individuals and is preceded by a sequence of precursor lesions such as chronic atrophic gastritis, epithelial hyperplasia, intestinal metaplasia and dysplasia, each of which have independently been linked to *H. pylori* infection and to an increased gastric cancer risk.[14, 15]

We have shown previously that the atrophic gastritis, hyperplasia and metaplasia associated with *H. felis* or *H. pylori* infection in C57BL/6 mice is predominantly an immunopathological response to the infection driven by Th1-polarized, IFN-γ-producing $CD4^+$ T-cells.[16, 17] Mice lacking functional $\alpha^+\beta^+$T-cells or the V(D)J recombination activation gene ($Rag1^{-/-}$) are protected from precancerous lesions. Moreover, the adoptive transfer of $CD4^+CD25^-$ effector T-cells from infected donors immunosuppressed recipients is sufficient to cause the development of precancerous lesions.[16] Furthermore we have shown that pharmacological suppression of T-cell activation and Th1 differentiation in the draining mesenteric lymph nodes prevents and even reverses gastric preneoplasia, implying that targeting T-cells during the infectious process may prevent the progression to gastric cancer.[17]

In addition to the pathological effects of the *Helicobacter*-specific immune response on the gastric mucosa, several lines of evidence indicate that the bacteria promote gastric carcinogenesis by jeopardizing the integrity and stability of their host's genome in multiple ways.[18] For example, *TP53* mutations are detected in more than 60% of advanced gastric cancers, as well as in gastric cancer precursor lesions: intestinal metaplasia (38%) and gastric dysplasia (60%).[19, 20] In addition, gastric cancers frequently show epigenetic silencing by promoter hypermethylation of genes involved in the maintenance of genetic stability: mismatch repair protein *MLH-1*[21, 22], *E-cadherin*[23] and the *p16* ^{INK4A} tumour suppressor gene.[24] Furthermore, gastric carcinogenesis may be supported by the fact, that the *Helicobacter*-infection leads to the reversible down-regulation of the mismatch repair (MMR) proteins MLH1, PMS1, PMS2, MSH2 and MSH6.[25,26] Furthermore, recent studies have reported elevated levels of 8-oxo-7,8 dihydroguanine (8-oxoG) in gastric cancer precursor lesions and gastric cancer.[27,28] 8-oxoG is an abundant DNA lesions causing G:C to T:A transversions that potentially lead to genome instability.[29] Izotti *et al.* also provide evidence for increased oxygen-induced damage in cancerous tissue. They show that the homozygous Ser326Cys polymorphism in the 8-oxoguanosine-glycosylase1 (*OGG1*) gene, encoding the enzyme removing 8-oxo-dG from DNA, leads to a significant increase in the occurrence of

oxidative DNA damage in *H.pylori* infected patients.[27] However, the exact mechanism of *Helicobacter*-induced carcinogenesis is poorly understood.

To maintain genome integrity, a variety of redundant pathways alerts the cell to the presence of DNA damage and coordinates an appropriate response. [30] DSB-induced signal transduction pathways include the DNA-dependent protein kinase (DNA-PK) and phosphatidylinositol 3-kinase related kinases (PIKKs) – ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR). The main DSB repair pathways are DNA non-homologous end joining (NHEJ) and homologous recombination (HR). The primary sensor of DSB is the MRE11-RAD50-NBS1 complex (MRN) which recruits ATM to the DSB, and is furthermore involved in the initiation of the feed back loop required for functional DNA damage repair (DDR).[31] Activated ATM or DNAP-PK in turn phosphorylate H2A histone family member X (H2AX) (γH2AX).[32] H2AX phosphorylation spans megabase-pair regions surrounding the lesion allowing their visualization as microscopically detectable foci.[33] γH2AX mediates the recruitment and retention of mediator proteins such as p53 binding protein-1 (53BP1), mediator of DNA damage checkpoint 1 (MDC1), breast cancer 1 (BRCA1) and the MRN complex at the site of DSB.[34] The second DSB-induced pathway, the ATR-dependent signal transduction pathway is activated in the process of DSB resection. In the absence of DSB, ATR is activated by single stranded regions of DNA that arise as a result of replication-fork stalling, or during repair of bulky lesions.[35]

In the present study, we examined the possibility that *H. pylori* infection directly leads to host DNA damage. We found that DNA double strand breaks (DSB) accumulate in various cell lines and in primary gastric epithelial cells upon infection with *H. pylori*. The fragmentation of host nuclear DNA requires infection with live bacteria that are in direct contact with their host cells. However the DSB occurred independently of the two *Helicobacter*-virulence factors VacA and the Cag pathogenicity island (CagPAI). We further observed that *Helicobacter*-induced DNA fragmentation triggers aDNA damage signaling response such as the phosphorylation of histone H2AX (γH2AX), and the recruitment of 53BP1 and MDC1 to sites of damage. Finally our data show that efficient DSB repair is only achieved upon bacterial eradication. The results obtained in this study indicate that *Helicobacter* drives tumorigenesis not only by causing an environment of chronic inflammation, but that *Helicobacter per se* possesses carcinogenic properties.

Results

H. pylori infection of cultured cells induces double strand breaks (DSB) in nuclear DNA that trigger a DNA damage signaling

To assess a possible effect of *H. pylori* infection on the integrity of host cellular DNA, we subjected infected cell lines to pulse field gel electrophoresis (PFGE). Hanada *et al*. adapted this technique to visualize fragmented DNA resulting from DSB.[36] The electrophoresis conditions were set as such that intact, high molecular weight genomic DNA could be separated from fragmented DNA. DNA fragments with sizes ranging from 0.5 to 2.5 megabases (Mb) are compacted in a single band which allows the detection and quantification of differentially sized and low abundant DNA fragments.[36] Infection of gastric adenocarcinoma cells (AGS) with *H. pylori* for 6h resulted in dose-dependent fragmentation of host cell DNA (Figure 1A) that persisted for at least 48 hours post infection (Supplemental Figure 1). The amount of *H. pylori*-induced DSB exceeded the DNA fragmentation resulting from 16h treatment with hydroxyurea (HU), a replication inhibitor that depletes cellular dNTP pools by blocking ribonucleotide reductase and was used here as a positive control (Figure 1A). Similar results were obtained in infected non-cancerous immortalized gastric epithelial cells (IMPGE) (Supplemental Figure 1). The massive DSB caused by the infection lead to complete inhibition of cell proliferation after 16h of infection, shown by blocked incorporation of the thymidine analogue, EdU (Supplemental Figure 2B).[37] We also tested the effect of *Helicobacter* infection in U2OS cells, an osteosarcoma cell line frequently used to study DNA damage signaling (Supplemental Figure 2A). Surprisingly, *H. pylori* adheres very well to these cells (Supplemental Figure 2C) rendering U2OS a suitable model to study *H.pylori* induced cellular DNA damage responses.

To assess the cellular response of infected cells to DSB, we monitored the cellular distribution of the 53BP1 and MDC1 upon infection. After phosphorylation of the H2A histone (H2AX), 53BP1 localizes to DSB, which is one of the earliest events of DSB processing and required for the activation of the ATM kinase.[38] Likewise, MDC1 is readily recruited to DSB where it binds to phosphorylated H2AX (γH2AX) and directs further recruitment of DNA damage response and repair factors to damaged chromatin.[39] The localization of 53BP1 and MDC1 at DSB is microscopically evident as foci. Indeed, *H.pylori* infection of AGS cells (Figure 1B, left panel) and U2OS cells (Figure 1B, right panel) leads to increased numbers of 53BP1 foci when compared to uninfected controls. All cells were positive for 53BP1 after 10Gy γ-irradiation (IR), and *H.pylori* infection leads to a 2-3-fold increase of 53PB1-positive cells (≥ 5 foci/cell). To assess whether this effect is restricted to cancer cells or if DSB also occur in primary gastric epithelial cells, we generated single cell suspensions of murine gastric epithelial cells. The cells were cultured on collagen-coated cover slips for two days and infected with *H. pylori* for 6h. Indeed, the number of 53BP1 positive cells was increased

also in primary cells upon *H.pylori* infection (Figure 1C). In addition to 53BP1 recruitment to DSB, we found that endogenous MDC1 also forms foci upon infection of U2OS cells (Figure 1E). The utility of U2OS cells as *in vitro H. pylori* infection model allowed us to confirm the above presented results but also to determine the kinetics of DSB foci formation in U2OS cells that stably express MDC1 as an eGFP fusion protein; eGFP-MDC1 was rapidly recruited to sites of DSB (Figure 1F) and the cells continued to show foci for at least 16h as determined by time lapse video microscopy (Supplemental Figure 1C). However, due to the long incubation and the motility of the cells, we could not determine if the same foci persisted or if the cell constantly acquired new DSB.

To rule out that the "fragmented DNA" detected by PFGE is of bacterial origin, we performed Southern Blot analysis of a pulse field gel comparing the signals on the PFG derived from bacterial concentrates with the signal intensities obtained from infected AGS cells. For that, we used a probe hybridizing to the *H. pylori* ribosomal 16S subunit (Supplemental Figure 2A). The r16S Southern Blot revealed that concentrated bacterial pellets showed a strong signal. In contrast, the band derived from infected cells, did not show a positive r16S signal (Supplemental Figure 2A). The results confirm that the band observed on the PFG in *Helicobacter*-infected cells is indeed fragmented host DNA and not of bacterial origin.

Since the process of apoptosis also leads to DNA fragmentation, we assessed the viability of the infected cells by immunoblotting for activated caspase-3 and cleaved PARP-1, as well as by flow cytometric analysis of hypodiploid cells and subG1 accumulation (Supplemental Figure 3B,C). No indication of apoptotic cell death was observed at 6, 16 and 54h post infection confirming that the infected cells are viable throughout the time of infection (Supplemental Figure 3B,C and data not shown). In conclusion, the presented data suggest that *H. pylori* efficiently causes DSB in various primary and transformed epithelial cells that leads to prompt recruitment of DNA damage response proteins to *Helicobacter*-associated DSB.

H. pylori-induced DSB depend on direct host-pathogen contact, but not on the Cag pathogenicity island and VacA virulence factors

To test whether DSB induction is specific to live *H. pylori*, we compared the potential of live and ethanol-killed bacteria to induce DSB. Furthermore, we infected cells also with the *E. coli* DH10B strain at high multiplicity of initial infection. Only live *H. pylori* efficiently induced DSB in AGS cells, whereas dead bacteria and *E. coli* caused no discernible effect on DNA integrity(Figure 2A). DSB induction by *H. pylori* further required a direct interaction between the bacteria and their host cells, as separation of the bacteria by a transwell filter abolished DSB induction (Figure 2B). We also assessed the potential of preconditioned

medium to induce DSB in untreated cells. Supernatants obtained from 16h infected AGS cells did not induce DSB in untreated cells, thus excluding the possibility that host or bacterial secreted substances account for DSB induction (Figure 2B). Finally we sought to determine whether well-known *H. pylori* virulence factors such as the CagPAI or VacA contribute to DSB induction. Isogenic mutants of G27 lacking the entire pathogenicity island or the gene encoding VacA induced DSB and 53BP1 foci to a similar extent as wild type bacteria (Figure 2C,D), suggesting that neither VacA nor the PAI-encoded type IV secretion system of *H. pylori* is essential for DSB induction. In summary, *H. pylori*, but not *E. coli* DH10B infection induces DSB in a contact dependent, but VacA- and CagPAI-independent manner.

Inhibition of ADP-ribosylation sensitizes cells to *H. pylori*-induced DSB

We have shown earlier that *H. pylori* infection induces poly-ADP-ribosylation of host cellular proteins.[40] Poly(ADP-ribose) polymerases (PARPs), are involved in the repair of various types of DNA damage[41, 42] by recruiting DNA repair proteins to the site of damage or by facilitating the access of the repair machinery to damaged DNA.[41,43] We have recently shown that PARP inhibitors such as PJ34 block *H. pylori*-induced PARP formation *in vitro* and prevent *Helicobacter*-associated gastric cancer precursor lesions *in vivo.*[40] PAR synthesis induced by *H. pylori* infection *in vitro* is dependent almost exclusively on PARP1, with a minor contribution of PARP2.[40] Both the formation of PAR and the induction of DSB are first detectable in infected cells between 3 and 6h p.i..[40] To assess whether ADP-ribosylation also plays a role in *H.pylori* associated DSB induction, we treated infected cell cultures with the small molecule inhibitor PJ34. Interestingly, PJ34 sensitized the cells to *H. pylori*-induced DSB at low multiplicities of infection (Figure 3B). This effect was further confirmed using other inhibitors of ADP-ribosylation (data not shown). The results suggest that PJ34 treatment possibly interferes with the ongoing DNA repair and thereby amplifies *H.pylori* induced DNA fragmentation at low MOIs.

***H. pylori*-induced DSB occur largely independently of reactive oxygen and nitrogen species**

It has been postulated that *Helicobacter*-induced reactive oxygen species are important drivers of carcinogenesis. In line with this hypothesis, mutations detected in chronically *H. pylori*-infected patients are predominantly ‘AT to CG’ and ‘GC to TA’ type transversions, and serve as indicators of oxidative DNA damage.[44] *H. pylori* induces reactive oxygen and nitrogen species (RONS) in AGS cells at MOIs which also trigger DSB (Figure 4A). To determine whether *H. pylori*-associated DSB induction is dependent on RONS, we utilized three complementary approaches in which the production of RONS was minimized

by growth under hypoxic conditions, pharmacological scavenging of RONS, or the combined targeted deletion of the genes encoding inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX2). AGS cells grown for 6 hours at 1% oxygen acquired more, rather than less, DSB upon infection with *H. pylori*. This observation suggests that the reactive oxygen is not the main mediator of *H. pylori*-induced DSB (Figure 4B). Similarly, addition of the RONS scavenger N-acetyl-cysteine (NA) to infected cells enhanced *H. pylori*-induced DSB rather than prevented them (Figure 4C); this result was particularly compelling as the same scavenger concentrations efficiently reduced H_2O_2-induced DSB (Figure 4C). Finally, infection of primary gastric epithelial cells isolated from iNOS/NOX2$^{-/-}$ mice and grown as described above (Figure 1C), induced 53BP1 foci (Figure 4D) and DSB (Figure 4E) to a similar extent as infection of wild type primary gastric epithelial cells, suggesting that the RONS produced through the combined activity of both enzymes are not essential for DSB induction. In summary, the results suggest that the RONS produced by gastric epithelial cells upon *H. pylori* infection are not mechanistically involved in DSB induction despite a temporal correlation of RONS and DSB induction.

The repair of *H. pylori*-induced DSB requires prior killing of *H. pylori*

Our data suggest that *Helicobacter*-infected continuously damages its host cell DNA and induces the activation of DNA damage signaling pathways as long as the bacteria are present. To test whether the cells are capable of initiating DSB repair upon killing of the bacteria, we infected AGS cells for 6h prior to 2 days of bacterial eradication therapy (ET). The efficient induction of DSB was verified after 6h of infection, as well as after 54h of continuous infection (Figure 5A). ET resulted in the efficient repair of DSB as judged by the disappearance of the band corresponding to broken DNA in the PF gel (Figure 5A) as well as the loss of repair-related 53BP1 and MDC1 foci (Figure 5B), indicating that *H. pylori*-induced DSB can be repaired by the affected cells, but only after *H. pylori* is inactivated.

Discussion

We show here that *H. pylori* efficiently induces DNA double strand breaks in primary gastric epithelial cells and various cancer cell lines. DSB formation depends on viable bacteria and direct bacterial contact with the host cells, but is independent of the Cag pathogenicity island and VacA, and also largely independent of host cellular ROS and RNS production. *H. pylori*-induced DSB can be repaired efficiently by the cells, but only upon elimination of bacteria. This observation may indicate a continuous induction of DSB by the bacteria and a subsequently lagging DSB repair process.

Very few reports are available on bacterially induced DNA damage of host cell DNA. Interestingly, of the few bacterial species suggested to possess genotoxic potential, all are

gastro-intestinal colonizers. Examples include *E. coli* which is commonly associated with gastrointestinal infections and *Campylobacter jejuni*, the most frequent bacterial cause of diarrhea in humans[45] have been associated with DSB induction. Recently, it has been shown that *E. coli* expresses a so-called peptide–polyketide compound (colibactin) that is essential for the induction DSB and subsequent ATM/H2AX activation.[46] The Cytolethal distending toxin (CDT) is another potential genotoxin found in *Campylobacter jejuni* and other bacteria (Thelestam, 2005) that is able to induce DSB in mammalian cells by the DNase activity of a CDT subunit (CdtB).[47] CDT is also expressed by *Helicobacter hepaticus* and other enteropathogenic bacteria. In murine *H. hepaticus* infection models, CDT is required for colonization,[48] mimics inflammatory bowel disease in humans by triggering a chronic intestinal inflammation,[49] and promotes hepatocellular dysplasia.[50] However, there is no evidence for CDT-dependent DSB induction in *H. hepaticus*, and *H. pyl*ori does not encode for a CDT-like protein. We suspect that *H. pylori* derived nucleases may take over the role of the CDT-DNase activity.

Furthermore we tested the effect of *Helicobacter*-induced reactive oxygen species on DSB induction. We show that DSB occurred also under low oxygen conditions and also in primary epithelial cells deficient for iNOS/NOX-2. The nitric oxide synthase (iNOS) and NADPH oxidase (NOX2) are important mediators of the pro-infalammatory response, microbial elimination and cancer development.[51, 52] Thus, inhibitors of those enzymes have been proposed for cancer therapy.[53] However, our results suggest that *Helicobacter*-induced ROS are not the only cause for DSB in the host cell DNA.

Quite surprisingly, we find that live bacteria not only induce DSB, but that the DSB persist as observed by PFGE and activation of DNA-damage signaling proteins. This observation may be explained in two different ways. It is possible, that the DNA repair machinery is either not functioning or not efficient enough to continuously repair newly established DSB. A second explanation includes that the *Helicobacter* caused arrest of cell proliferation may result in an impaired efficiency to synthesize DNA repair proteins required for DSB resolution. This finding extends previous reports showing that *H. pylori* down-regulates the components of the host's mismatch repair machinery, MSH2, MSH6, MLH1 and PMS2, both at the protein and RNA levels.[54] Follow-up work by Machado *et al.* showed that this down-regulation indeed results in a decreased repair efficiency of infected compared to uninfected AGS cell extracts of 2bp loop heteroduplex substrates.[55] The same authors also reported increased genetic instability of three loci carrying CA repeats in mice chronically infected with *H. pylori*;[55] this finding may be relevant as microsatellite instability is a common feature of sporadic gastric cancer [56] and MMR levels tend to recover in patients treated for the infection by antibiotic eradication therapy [25]. Similarly, certain components of the BER machinery, such as APE2, were found to be downregulated upon infection of gastric

epithelial cells[55] and this may correlate with an increased susceptibility of BER-deficient mice lacking the enzyme alkyladenine DNA glycosylase to *Helicobacter*-induced gastric cancer precursor lesions.[57] Whether the persistence of DSB benefits the bacteria in terms of colonization or persistence remains to be clarified. Important to note is that *H.pylori*-induced DSB only occur at very high infection doses and even then, only approximately thirty percent of the cells show signs of DSB-induced DNA damage signaling.

However, when bacteria are eradicated, the cells are able to repair the DSB caused by the infection. Interestingly, this process requires 48 hours to be observed by PFGE, suggesting that *Helicobacter*-induced DSB are of rather high complexity or that intracellular bacterial factors have a long half life. The above presented data suggest that Helicobacter-induced DSB can be resolved by the host DNA repair machinery, however.

In conclusion, we propose that persistent *H. pylori* infection causes DSB induction in the infected host cells, thereby contributing to the genetic instability and frequent chromosomal aberrations and cancer development.

Figures 1-5 and Supplemental Figures 1-3

Figure 1

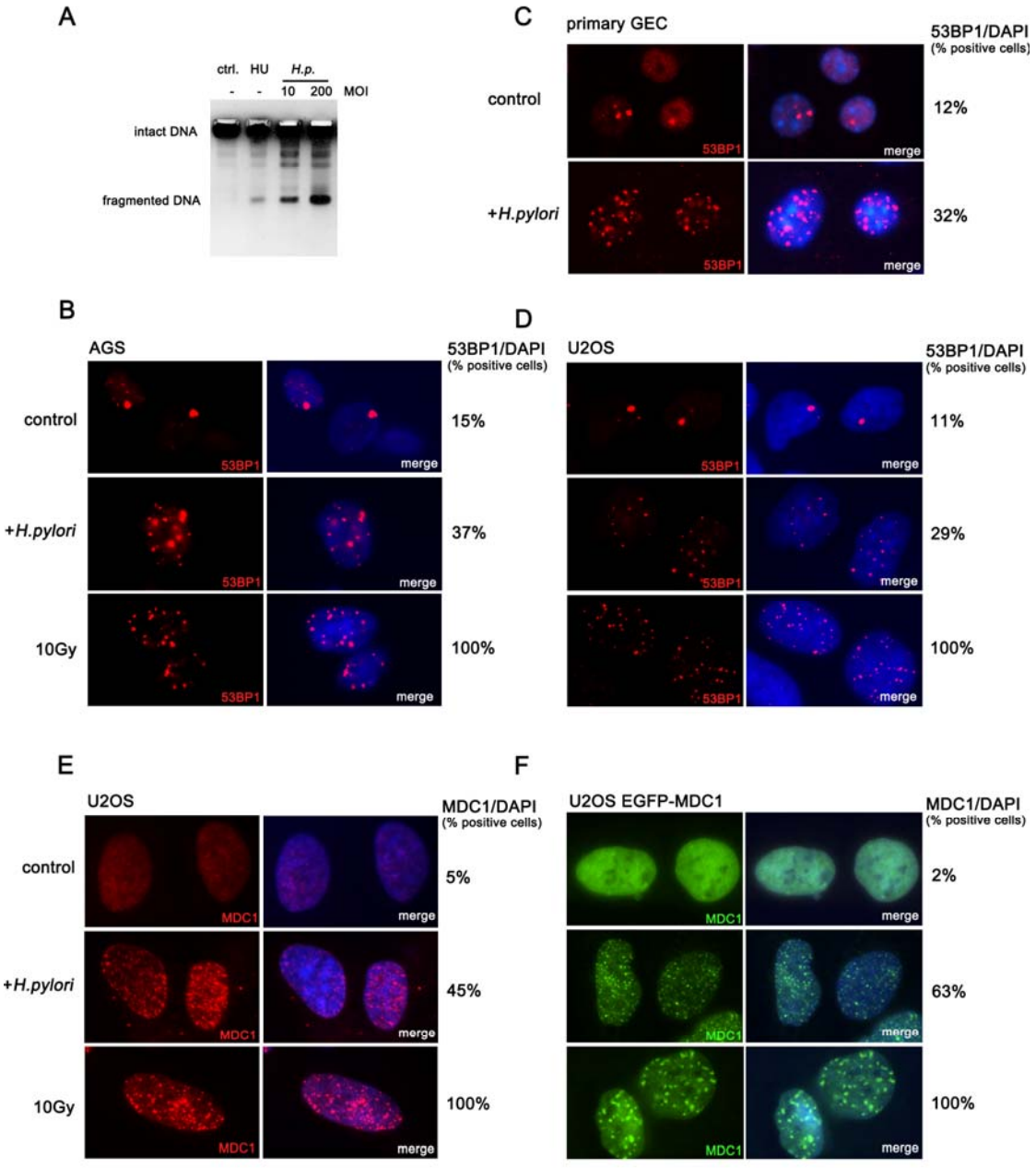


Figure 1. *H. pylori* infection of cultured cells induces DNA double strand breaks (DSB) followed by the activation of DNA damage response pathways

A, AGS cells were infected with *H. pylori f*or 6h at multiplicities of infection (MOI) of 10 and 200. 5mM Hydroxyurea (HU) treatment for 16h served as positive control. DNA integrity was assessed by pulse field gel electrophoresis (PFGE). Fragmented DNA accumulates in the band comprising fragments from 0.5-2.5Mb. B, AGS cells were infected with *H. pylori* for 6h

and stained for 53BP1, an early marker of DSB; Cells irradiated with 10Gy served as positive control. In C, explanted primary gastric epithelial cells from wild-type C57BL/6 mice and in U2OS cells (D) were infected for 6h with *H. pylori* and immunostained for 53BP1; Cells irradiated with 10Gy served as positive control. Cells with more than 5 foci per nucleus were graded as positive and are represented in percentages of total cells counted ($53BP1^+$/number of $DAPI^+$ cells). In D, AGS cells were infected with *H. pylori* for 6h and stained for MDC1. E, eGPF-MDC1-expressing U2OS cells were infected and eGFP-MDC1 foci formation was quantified.

Figure 2

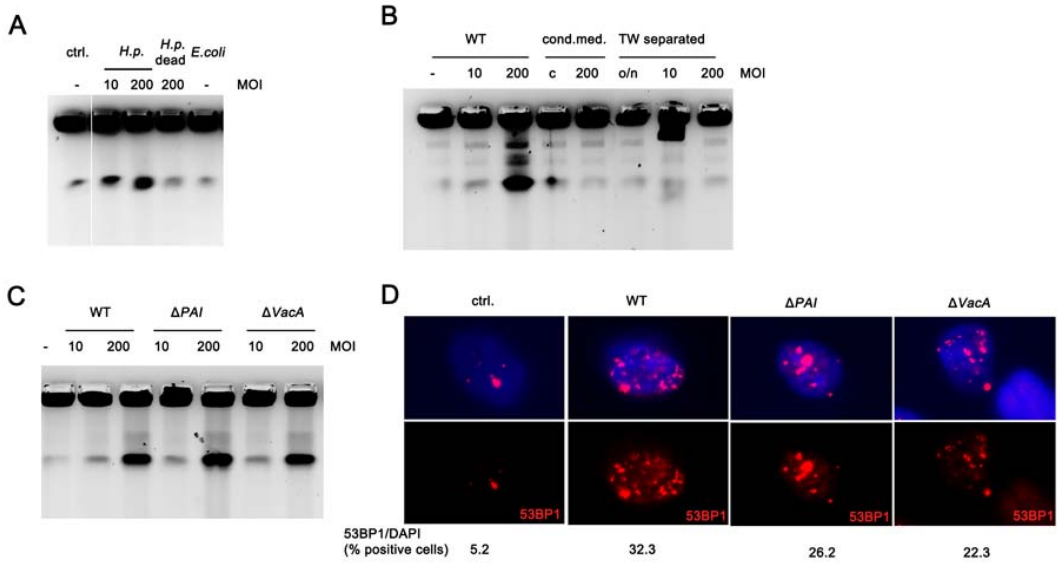


Figure 2: DSB are *H. pylori*-specific and depend on living bacteria and direct bacterial contact with the host cells.

A, AGS cell were co-cultured with live *H. pylori* (MOI 200), dead bacteria (MOI 200) or *E. coli* DH10B (MOI 1000) or left uninfected. DNA fragmentation was assessed by PFGE. B, DNA fragmentation was assessed by PFGE of: AGS cells that were left uninfected or were infected with *H. pylori* for 6h at MOI 200 (lanes 1-3). In lanes 4 and 5, cells were treated with sterile-filtered cell culture supernatants obtained from 16h control- or *H. pylori*-infected (MOI 200) AGS cells. In lanes 6-8, trans-well membranes were used to physically separate AGS cells from a concentrated over-night *H. pylori* culture supernatant (lane 6), or living bacteria at MOIs 10 (lane 7) and 200 (lane 8). In C+D, AGS cells were infected with wild-type *H. pylori* or isogenic mutants lacking the Cag pathogenicity island (Δ*PAI*) or the vacuolating cytotoxin A (Δ*vacA*). DNA fragmentation was assessed by PFGE (C) and 53BP1 immunostainings (D). Cells with more than 5 foci per nucleus were graded as positive and are represented in % of total cells counted ($53BP1^+$/number of $DAPI^+$ cells).

Figure 3

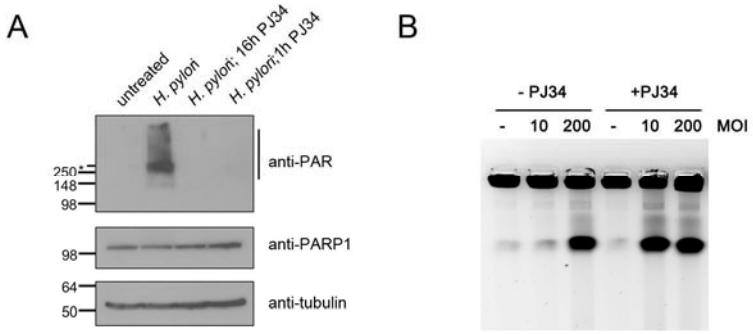


Figure 3. Inhibition of ADP-ribosylation leads to hypersensitivity of infected cells to DSB

A, AGS cells were left untreated, were infected with *H. pylori* at an MOI 200 or were infected and treated with 15µM PJ34. PJ34 treatment started either at the time of infection (24h) or 1h prior to harvesting (1h). Immunoblots against PAR and PARP1 are shown. B, AGS cells were infected with *H. pylori* at the indicated MOIs for 6h or were infected and treated with 15µM PJ34 during the entire time of the infection. DNA fragmentation upon infection is visualized by PFGE.

Figure 4

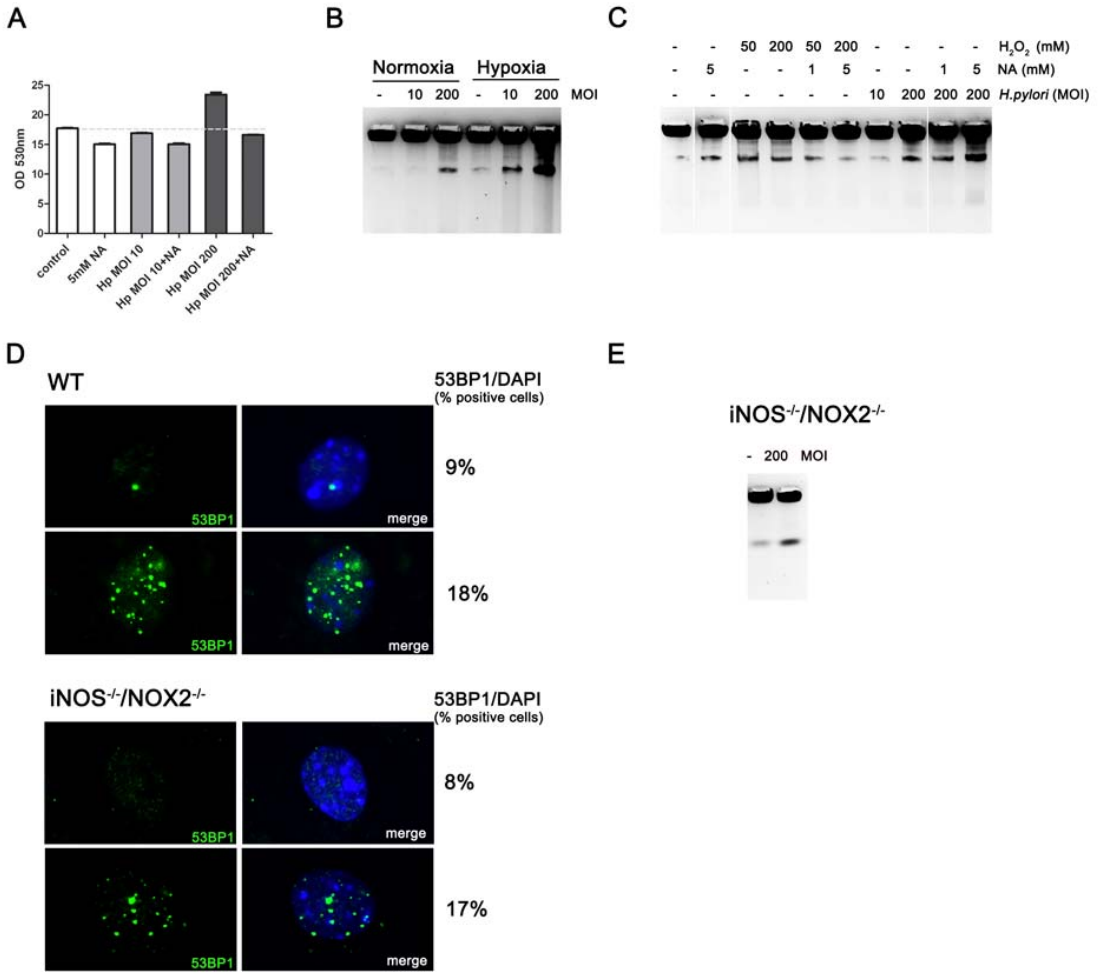


Figure 4. *H. pylori*-associated DSB occur largely independently of reactive oxygen species.

A, AGS cells were left untreated, were treated with 5mM N-acetyl-cysteine (NA), a radical scavenger and/or were infected with *H.pylori* at MOIs 10 and 200. The relative induction of reactive oxygen species (ROS) was assessed by oxidation of 2,7-Dichlorofluorescein Diacetate (DCF-DA) to fluorescent DCF (emission at 530nm) and was quantified in a microplate reader. B, AGS cells were infected with *H. pylori* for 6h at the indicated MOIs at 20% (normoxia) and 1% (hypoxia) oxygen in a gas-controlled glove box. DNA fragmentation is visualized by PFGE. C, AGS cells were left untreated, were treated with 5mM NA and/or were infected with *H.pylori* at MOIs 10 and 200. Cells treated with 50 and 200mM H_2O_2 served as positive controls. DNA fragmentation was assessed by PFGE. D, AGS cells were infected at the indicated MOIs for 6h. In addition, NA was added at 1 and 5mM where indicated. Hydrogen peroxide (H_2O_2) treatment for one hour with or without NA treatment served as positive control. DNA fragmentation is visualized by PFGE. D, Primary gastric epithelial cells explanted from wild-type C57BL/6 mice and mice lacking iNOS (iNOS$^{-/-}$) and NADPH peroxidase 2 (NOX2$^{-/-}$) (iNOS$^{-/-}$ NOX2$^{-/-}$) were infected with *H. pylori* for 6h. Cells

were immunostained with 53BP1. Cells with more than 5 foci per nucleus were graded as positive and are represented in % of total cells counted ($53BP1^+$/number of $DAPI^+$ cells). E, Primary gastric epithelial cells explanted from $iNOS^{-/-}$ /$NOX2^{-/-}$ mice were infected for 6h with *H. pylori* at the indicated MOI. DNA fragmentation upon infection is visualized by PFGE.

Figure 5

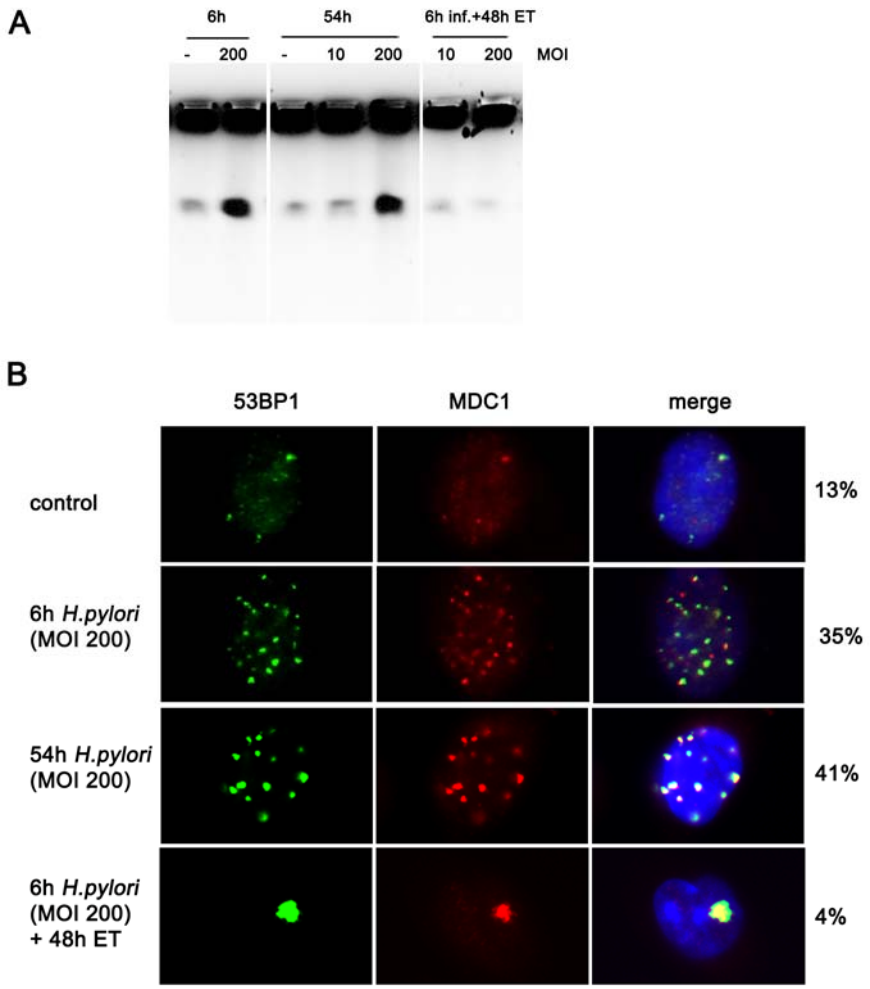
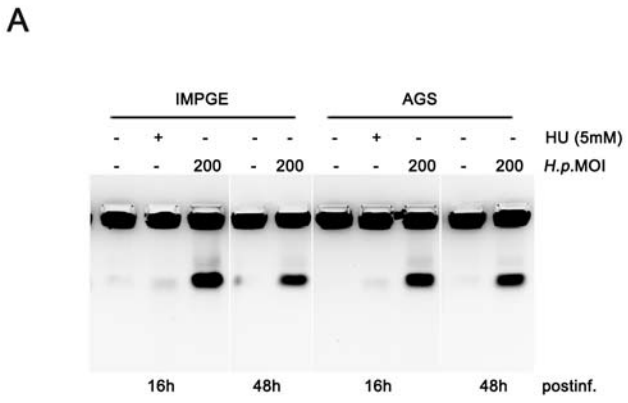


Figure 5. *H. pylori*-induced breaks are repaired only after bacterial killing

A, AGS cell were infected with *H. pylori* at MOI 200 for 6h. Cells were washed and the remaining bacteria were killed with 4.5µg/mL metronidazole, 10µg/mL tetracycline hydrochloride and 1.2µg/mL bismuth subcitrate (eradication therapy, ET) and grown in full medium containing penicillin/streptomycin for 48 hours to allow DNA repair to occur. On the second day, ET treatment was repeated. After 54h hours of infection and/or 48h ET treatment, cells were harvested and subjected to PFGE. B shows 53BP1 immunostaining of the cells described in A. Cells with more than 5 foci per nucleus were graded as positive and are represented in % of total cells counted ($53BP1^+$/number of $DAPI^+$ cells).

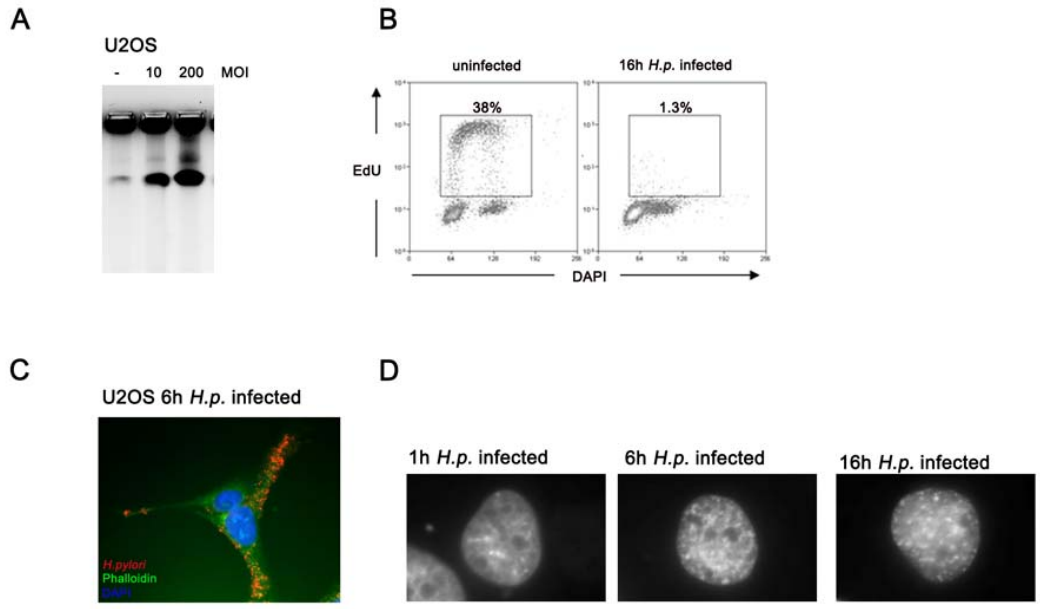
Supplemental Figure 1



Supplemental Figure 1: *H. pylori* induces persistent DSB also in primary immortalized gastric epithelial cells

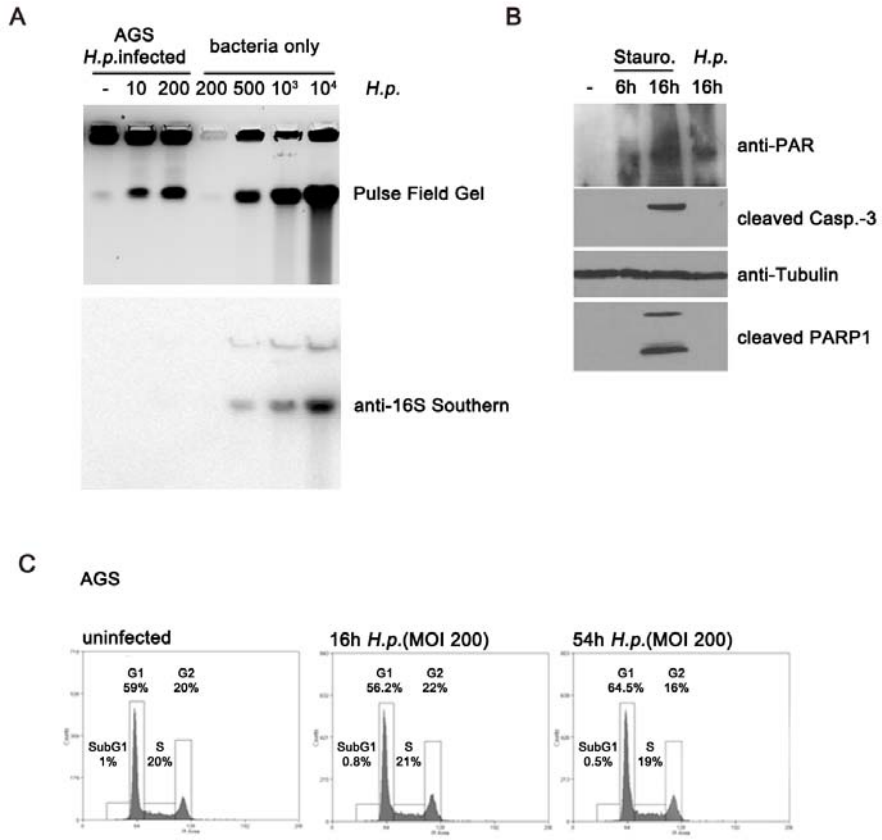
A, Immortalized gastric epithelial cells (IMPGE) and AGS cells were infected for 16 and 48 hours with *H. pylori* at the indicated MOIs. 16h 5mM Hydroxyurea (HU) treatment served as positive control. DNA fragmentation was visualized by PFGE.

Supplemental Figure 2



Supplemental Figure 2: U2OS cells can be used as a model for *H. pylori*-induced DSB
A, U2OS (osteosarcoma) cells were infected with *H. pylori* for 6h at the indicated MOIs. DNA fragmentation was assessed by PFGE. B, U2OS cells were infected with *H.pylori* for 16h. Proliferating cells were pulse-labelled with EdU for 20min. EdU positive cells were quantified by FACS. C, U2OS cells were infected with *H. pylori* for 6h. Immunofluorescence images showing *H. pylori* (red), Phalloidin (green) and nuclei (blue) are presented. D, U2OS-eGFP-MDC1 cells were infected with *H. pylori* and MDC1-foci formation was monitored over a period of 16h. Pictures of single cells were taken at 1h, 6h and 16h post infection.

Supplemental Figure 3



Supplemental Figure 3: Fragmented DNA indicates DSB and neither corresponds to bacterial DNA nor to apoptotic DNA damage

A, AGS cells were infected with *H. pylori* for 6h at the indicated MOIs. In addition, bacterial pellets corresponding to MOI 200, 20000 and 10000 were loaded. Fragmented DNA and bacterial DNA were visualized by PFGE (upper panel). The DNA was transferred to a Zeta membrane, UV-cross-linked and probed for *H. pylori* using a dCTP α^{32}-P labeled 16S rRNA probe (lower panel). B, AGS cells were infected with *H. pylori* for 16h or were treated for 6 and 16h with 5µM staurosporine, a global tyrosine kinase inhibitor known to induce apoptosis. Immunoblots are shown for the cleavage products of PARP1 and Caspase-3. Tubulin served as loading control. C, AGS cells were infected with *H. pylori* for 16 and 54h. Cell cycle distribution was assessed by propidium iodide (PI) staining. The percentage of cells in the respective cell cycle phases is indicated.

Materials and Methods

Mice, bacteria, cell lines and infections

C57BL/6, (Charles River Laboratories, Sulzfeld, Germany) and iNOS-/-/NOX2-/- mice were used to generate primary gastric epithelial cell cultures. *H. pylori* was grown on solid medium on horse blood agar containing 4% Columbia agar base (Oxoid; Basingstoke), 5% defibrinated horse blood (HemoStatLabs), 0.2% β-cyclodextrin, 5µg/ml trimethoprim, 8 µg/ml amphotericin B, 10µg/ml vancomycin, 5µg/ml cefsulodin, and 2.5 U/ml polymyxin B sulfate (all from Sigma-Aldrich) at 37°C for 2 days under microaerophilic conditions. For liquid culture, *H. pylori* was grown in Brucella broth (Difco) containing 10% FBS (Life Technologies) with shaking in a microaerobic atmosphere at 37°C. AGS cells (ATCC CRL 1739; human gastric adenocarcinoma epithelial cell line) and U2OS cells and U2OS-EGF-MDC1 and primary immortalized gastric epithelial cells (IMPGE) (Sayi , 2009) and were infected with *H. pylori* strains G27 or isogenic mutants lacking the Cag pathogenicity island (PAI), G27ΔPAI [58] or the vacuolating toxinA (VacA) G27ΔVacA [59] for 6h to 54 hours with indicated multiplicities of infection (MOI). Primary gastric epithelial cells were generated as follows: stomach was excised, washed in PBS and digested for 7min in 20mM ETDA/Hanks Balanced Salt Solution. Stomach was grinded between two glass plates and cell suspension seeded into rat-tail collagen (50 µg/ml; C3867 Sigma) coated cell culture dishes in 1:1 Ham's F12/DMEM medium containing Penicillin/Streptomycin and FBS (Life Technologies). The DH10B *E.coli* strain was used at MOI 1000. Experiments under hypoxic conditions (1% oxygen) were performed in a gas-controlled glove box (InVivO2 400, Ruskinn Technologies, Leeds, UK).

Immunostainings and Immunoblots

For immunostainings, cells were fixed in methanol and stained with 53BP1 (H300: sc-22760, Santa Cruz; 1/500), MDC1 (3835; 1/200; courtesy of PD Dr. M. Stucki, Zurich)[60], γH2AX (Upstate: 05-636; 1/500), anti-*Helicobacter Pylori* (polyclonal; Dako; 1/200), Phalloidin-Alexa 488 (Molecular Probes) and mounted with vectashield (Vector Laboratories). Cells were imaged in a Leica Leitz DM RB microscope equipped with a Leica DFC 360 FX camera. Images were taken at 60 to 100x magnification using the Leica Application Suite 3.3.0 software. At least 100 nuclei were assessed and graded positive if more than 5 foci were detected per nucleus. PARP1 and PAR expression was analyzed by immunoblotting of cell extracts using rabbit anti-PARP1antibody H-250 (Santa Cruz) and anti-PAR antibody (no. 551813, BD Biosciences) respectively. As markers of apoptosis, PARP1 (sc7150) and Caspase-3 were used.

si/shRNAs

The following proprietary Qiagen siRNAs were used: PARP1 mouse: Mm_Parp1_4 HP siRNA (NM_007415); PARP1 human Hs_PARP1_6 (NM_001618); PARP2 mous: Mm_Parp2_6 HP siRNA (NM_009632); PARP2 human: Hs_PARP2_6 HP (NM_001042618, NM_005484) E

Detection of ROS with 2,7 Dichlorofluorescein Diacetate

Cells were washed and 5µM 2,7 Dichlorofluorescein Diacetate (DCF-DA, Sigma) in PBS was added to the cells and incubated for 30min at 37°C protected from light. Oxidation-induced de-esterified fluorescent DCF was detected at 530nm.

Cell cycle analysis

For cell cycle analysis, cells were fixed in 70%ethanol followed by a 100ug/ml RNase treatment and staining with 25ug/ml propidium iodide. Cell-cycle distribution was measured by flow cytometry in a Cyan9 instrument (Beckman Coulter) and analyzed using the Summit Software (Beckman Coulter).

Transwell-Assay, pharmacologic treatments with PJ34 and scavengers

0.4um cell culture inserts (Falcon 353493) were used to separate bacteria or supernatants from cells. PJ34 was added as PJ34 (N-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide, Enzo Life Sciences) was used at 15uM in cell culture and Hydroxy Urea (H8627, Sigma) was used at 5mM, N-acetyl cysteine (Sigma) was used at 1 and 5mM and staurosporine at 5uM (S5921, Sigma). Bacteria were killed with penicillin/streptomycin combined with with 4.5 mg/mL metronidazole, 10 mg/mL tetracycline hydrochloride (both Sigma-Aldrich), and 1.2 mg/mL bismuth subcitrate (Park-Davis).

Pulse Field Gel Electrophoresis

This protocol was adapted from Hanada et al., 2007.[36] Briefly, cells were infected with H.pylori, trypsinized and immobilized in a 2% agarose plug ($5x10^5$ cells/plug). Plugs were incubated in lysis buffer (100 mM EDTA, 1% (w/v) sodium lauryl sarcosyne, 0.2% (w/v) sodium deoxycholate, 1 mg ml–1 proteinase K) at 37 °C for 48 h and then washed them in TE buffer (10 mM Tris-HCl (pH 8.0), 100 mM EDTA) before loading them onto an 0.9% agarose gel. Electrophoresis was performed for 23 h at 14°C in 250 mM Tris-borate with EDTA (TBE) using a Biometra Rotaphor apparatus with the following parameters: voltage 180–120 V log; angle from 120° to 110° linear; interval 30 s to 5 s log. Gel was stained with EtBr and analyzed using an Alpha Innotech Imager.

Southern Blot analysis

Anti-*Helicobacter spp*.-specific16S probe was generated using primers previously published by Harper *et al*.: C97 forward: GCTATGACGGGTATCC; C05 reverse: ACTTCACCCCAGTCGCTG resulting in a 1200 bp fragment.[61] Pulse Field Gel electrophoresis was performed as described above. Gel was stained and UV cross-linked with 150mJ, probes transferred to Zeta-Probe GT nylon membrane for 12hours and probed with dCTP α^{32}-P labbleled 16S probe (Roche #11004760001) followed by exposure in a Typhoon 9400 PhosphoImager.

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3.3 Article contributions

3.3.1 TLR2-activated B-cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing Tr1 cells *(manuscript submitted)**

*For the full text article refer to Appendix Section.

Authors: Ayca Sayi, Esther Kohler, Isabella M Toller, Richard A. Flavell, Werner Mueller, Axel Roers and Anne Mueller

Journal: Journal of Immunology

Contribution: Experimental assistance for adoptive transfer experiments, discussion of the manuscript

Summary: This manuscript provides evidence that excessive Helicobacter-induced Th-1 driven gastric immunopathology is restricted by cooperating 'regulatory' B- and T-cells. Using mouse strains with defined defects in either of the two populations, we found that B-cells, upon their MyD88-dependent activation by *Helicobacter* TLR-2 ligands, are able to efficiently induce IL-10-dependent suppressive activity in conventional $CD4^+$ T-cells, converting these into T regulatory-1 (Tr-1)-like cells. The cooperative activity of 'regulatory' B-cells and Tr-1 cells prevents the development of gastric premalignant lesions, thus providing evidence that B-cells can have important immunomodulatory function during immune responses to persistent bacterial infections.

4. Discussion

Gastric cancer is among the leading causes of cancer-related deaths worldwide due in large part to the inefficient diagnosis and therapy of premalignant lesions.[262] In this study we provide strong evidence that targeting the $CD4^+$ T-cell mediated immune response by inhibiting COX-2 and PARP enzymatic activities prevents and sustainably cures *Helicobacter*-induced gastric pre-neoplastic lesions in the murine stomach. We show that the protective effect is procured by blocking the effector functions of pathogenic $CD4^+IFN\gamma^+$ T-cells. Furthermore we show that *Helicobacter* infection leads to DNA fragmentation and triggers DNA damage signal transduction pathways characteristic of DNA double strand breaks (DSB), suggesting a direct role of *Helicobacter* in cancer development.

In the following section, the importance of pathogenic $CD4^+CD25^-$ effector T-cells will be discussed in the light of recent findings from our and other laboratories on the effects of vaccination and tolerance induction on gastric preneoplastic pathology. This section will be followed by an illustraton of the results obtained in this study and put into perspective with known concepts combined with future prospects and therapeutic implications.

4.1 Pathogenic $CD4^+CD25^-$ T-cells Mediate *Helicobacter*-Associated Disease in the CD57BL/6 Mouse Model

Patient data as well as mouse and gerbil models have impressively shown that *Helicobacter*-induced chronic inflammation is an essential prerequisite for developing gastric cancer.[36, 57, 58, 70, 71, 263] Infection of C57BL/6 mice with *Helicobacter pylori* and the close relative *Helicobacter felis* results in a strong pro-inflammatory and adaptive immune response that is reminiscent of the human setting.[17, 264]

The *Helicobacter*-induced inflammation is mediated by T helper (Th) cells. Th responses have either predominant Th1 (mainly cell mediated) or Th2 (mainly antibody dependent; humoral) character. Typical Th1 cytokines are IFN-γ, TNF-α, IL-12 and IL-2; Th2 cells predominantly secrete IL-4 and IL-5. *Helicobacter* infection triggers both responses, although gastric mucosal cytokine profiles show an over-representation of Th1 type cytokines.[17, 265, 266] Those cells are closely associated with the development of *Helicobacter*-induced gastric pathogenesis.

As a counter balance to inflammation-promoting effector T-cells, immunosuppressive regulatory T-cells (Treg) play a crucial role during *Helicobacter* infection. Tregs mediate their regulatory effects by secreting the immunosuppressive cytokines IL-10 and TGFβ.[267] Tregs function to avoid exaggerated immune responses to the infection, but thereby also facilitate a state of chronic infection. The importance of Tregs is reflected by our and other laboratories' data showing that *Helicobacter* induces more severe pre-neoplastic lesions and fails to

persistently colonize IL-10-deficient mice or mice lacking $CD4^+CD25^+$ Treg[268] or $CD25^+FOXP3^+$ Treg.[269]

Our hypothesis that *Helicobacter*-associated disease is indeed the result of an immunopathological response of the gastric mucosa is corroborated by the fact that *Helicobacter*-infected mice lacking cellular immune responses, e.g. RAG-$1^{-/-}$ (B- and T-cell-deficient) or TCR-$\beta^{-/-}$ (T cell-deficient) mice, are highly colonized but do not develop gastric pathology.[270] This phenotype can be rescued by the adoptive transfer of $CD4^+IFN\text{-}\gamma^+$ effector T-cells, which results in severe gastritis in *H. pylori*-infected recipients. Conversely, co-transfer of Tregs antagonizes the T-effector functions and blocks the development of gastric cancer precursors.[271, 272]

IFN-γ is an important mediator of inflammatory diseases and is critically involved in anti-microbial responses. This is underscored by observations that IFN-γ deficient/inhibited mice develop less severe symptoms of inflammation and epithelial atrophy.[18, 273-275] We have shown previously that IFN-γ expression significantly correlates with the appearance of epithelial atrophy and hyperplasia.[18] Furthermore, we noted an inverse relationship between IFN-γ expression and the number of colonizing bacteria in the stomach. Interestingly and in accordance with these results , IFN-$\gamma^{-/-}$ fail to reduce the bacterial burden and can not resolve bacterial colonization as efficiently as wild-type mice in a vaccination model.[18] The effect of IFN- γ on bacterial reduction is most likely of indirect nature, since IFN-γ has been shown to potentiate the leukocyte/macrophage-mediated uptake and killing of various bacterial strains.[276-278]

We could further demonstrate that $CD4^+$ T-cells are the prime source of pathogenic IFN-γ in our model, since adoptive transfer of $CD4^+CD25^-$ from IFN-$\gamma^{-/-}$ mice did not cause epithelial pathology in *Helicobacter*-infected immunodeficient mice (TCR$\beta^{-/-}$ and Rag$2^{-/-}$).[18] Our data support a dual role of IFN-γ during *Helicobacter* infection. As a pro-inflammatory cytokine it fights bacterial colonization, but its prolonged expression is also strongly correlated with increased severity of epithelial atrophy in infected mice. A role of IFN-γ in the progression of pre-neoplastic conditions is supported by the observation that recombinant IFN-γ treatment of gastric epithelial cells results in mucous neck cell hypertrophy.[75] Another study observed that a rare progenitor cell type of the antral gland that possesses multilineage potential multiplies in response to IFN-γ and thus may contribute to preneoplasia.[279]

Another important cytokine during *Helicobacter* infection is the Th1 suppressing interleukin IL-10. A mentioned above, IL-10 is secreted by Treg, but also other immune cells such as dentritic cells (DCs).[280] *Helicobacter* infection of IL-10-deficient mice leads to rapid induction of gastric preneoplasia and efficient bacterial clearance. Data generated in our lab further show that IL-10 proficiency is important for the regulatory function of T-cells, since mice with a $CD4^+$ T-cell specific deletion of the *il-10* gene display accelerated development of

epithelial pathology and lower bacterial colonization as compared to IL-10-proficient control mice.[272] In a very recent study conducted in our lab, we show that a Treg-biased Treg/Teffector ratio confers peripheral immune tolerance in neonatally *Helicobacter*-infected mice that is characterized by high bacterial colonization but absence of epithelial pathology compared to mice that were infected as adults.[281] Neonatally infected mice fail to upregulate Th1 cytokines (e.g. IFN-γ, IFN-γ inducible protein 10 (IP10) and MIP2) despite normal T-effector development. However, if Treg are depleted by various means or are not able to respond to TGFβ signalling, neonatally infected mice fail to mount and/or maintain tolerance.[281] IL-10-deficient mice can be tolerized initially, but do not stably maintain neonatally-induced tolerance to *Helicobacter* infection.[281]

$CD4^+$ T-cells are not restricted to hyperplastic and metaplastic lesions preceding gastric adenocarcinoma but also play important roles in the development and maintenance of another type of *Helicobacter*-associated gastric malignancy, i.e. mucosa-associated lymphoid tissue (MALT) lymphoma. We have recently shown that tumor-derived $CD4^+$ T-cells strongly stimulate tumor B-cell proliferation, supporting their essential contribution to MALT lymphomagenesis.[282] The importance of T-cells in inducing and/or maintaining MALT lymphomas is underlined by the observation that $CD4^+$ T-cell and $CD4^+CD25^+$ Treg depletion led to tumor regression.[282] In addition, we observed CCL17/22-dependent recruitment of highly suppressive Treg ($CD4^+CD25^+FOXP3^+$) to these B-cell tumors. This implies an essential role of *Helicobacter*-induced T cells in MALT lymphomagenesis and its maintenance. This observation together with the beneficial effect of bacterial eradication in MALT lymphoma highlights the antigen dependency of this B-cell malignancy.[282] In other words, effector and regulatory $CD4^+$ T-cells regulate not only the intestinal type of gastric cancer initiation and progression, but are also master regulators of MALT lymphomagenesis. Targeting this cell population at early stages of gastric carcinogenesis may offer the possibility to treat and prevent progression from benign gastric cancer precursors to malignant lesions.

4.2 Activation of COX-2/PGE_2-Dependent Pathways Prevents and Cures *Helicobacter*-induced Gastric Preneoplastic Lesions

COX-2/PGE_2-dependent pathways play central roles in the host's response to inflammatory stimuli and critically shape cellular immune responses.[1] Moreover, COX-2 is also implicated in malignant transformation of cells by promoting proliferation, migration and invasion and by inhibiting apoptosis.[283] We provide experimental evidence that COX-2/PGE_2 dependent signalling plays an essential immunosuppressive role during early stages of *Helicobacter*-associated disease by inhibiting pathogenic Th1 immune responses. We show that activation of this pathway by administration of exogenous, synthetic PGE_2 prevents, and COX-2 inhibition exacerbates, *Helicobacter*-induced atrophic gastritis, epithelial hyperplasia and metaplasia. Data obtained from *in vitro* studies strongly suggest that PGE_2 exerts an immunosuppressive effect on T-cells, especially $CD4^+IFN\text{-}y^+$ cells, which fail to migrate to the stomach and to prime other T-cells in the mesenteric lymph nodes. PGE_2 treatment of anti-CD3/28 stimulated T cells further resulted in a complete block of IL-2 and IFN-γ expression and thus proliferation, and inhibited up-regulation of the high-affinity IL-2 receptor α chain.

The contribution of *Helicobacter* infection to gastric cancer development is a proven concept and bacterial eradication therapy (ET) has been associated with beneficial effects, for example in patients with gastric atrophy.[262] However, increasing recent evidence contradicts the efficacy of ET in the reversal of intestinal metaplasia and the prevention of cancer progression,[262, 284, 285] and highlights the need for alternative treatment options. PGE_2 treatment may constitute a suitable new therapeutic alternative to prevent and reverse *Helicobacter*-induced pre-existing lesions.

We and others show that *Helicobacter* infection leads to up-regulated COX-2 and PGE_2 expression in infected cell lines and in the stomach of infected mice.[88, 118] However, the role of COX-2/PGE_2 signalling in precancerous lesions has only poorly been described to date. Therefore we assessed the effects of inhibition of COX-2 enzymatic activity during *H.felis* infection using the COX-2 inhibitor Celecoxib. COX-2 inhibitors have been known to efficiently prevent the occurrence of sporadic colon cancer and other cancers of the gastrointestinal tract (GI) for some decades[5, 139, 140, 143] by abolishing the immunosuppressive role of PGE_2 and thereby rendering the tumor accessible to the host's immune defence.[135, 286]

However, COX-2 inhibition during early stages of inflammation-induced maligancies shows inverse effects. COX-2 inhibition at very early time points of infection led to significantly aggravated inflammation and epithelial atrophy in treated compared to untreated mice. In accordance with this observation, activation of COX-2 dependent signalling by exogenous PGE_2 treatment during infection resulted in abrogated inflammation and absence of epithelial atrophy. This notion suggests that the absence of PGE_2's immunosuppressive function during acute inflammation leads to uncontrolled immune cell proliferation and

accelerated development of *Helicobacter* associated epithelial atrophy. Our results are supported by various previous studies reporting exacerbated gastritis in *H. pylori*-infected rodents under conditions of COX inhibition or deletion of *COX* genes.[82, 287-290] Furthermore, an experimental 45-day treatment of *Helicobacter*-infected individuals with aspirin, a non-steroidal anti-inflammatory drug (NSAID) and unspecific COX inhibitor, resulted in significantly elevated gastric epithelial atrophy compared to uninfected counterparts.[291] However, conventional NSAIDs target all COX isoforms and are known to cause adverse gastrointestinal and renal complications that occur irrespective of *Helicobacter* infection.[292]

The COXibs, a new generation of anti-inflammatory drugs that specifically target COX-2 have shown significantly less gastrotoxicity in clinical trials than conventional NSAIDs. Several of these new compounds later had to be withdrawn from the market due to increased cardiovascular toxicity observed in clinical trials with refecoxib and celecoxib.[293, 294] Thus, the chemopreventive usage of COXibs is questionable, and further down-stream targets probably constitute safer target candidates.[295]

In this study we show that COX-2 inhibition results in two inversely related effects in the context of *Helicobacter*-associated carcinogenesis. At initial stages of the infection, when superficial and atrophic gastritis are the predominant precancerous lesions, the inhibition of COX-2 signalling results in the block of COX-2/PGE_2-mediated immunosuppressive and thus protective effects on the gastric mucosa. Impaired COX-2/PGE_2 functions thus lead to an uncontrolled pro-inflammatory response and hence more severe disease symptoms. By contrast, abrogated immunosuppression in a tumor restores a tumor-specific immune response and subsequent tumor regression.[1, 142] It would be interesting to test in our model if COX-2 inhibtion supports cancer progression if administered once precancerous lesions have formed.

Strikingly and in accordance with the above presented data, we have proven that exogenous PGE_2 has beneficial effects in infected mice. We could show that PGE_2 treatment not only prevented *Helicobacter*-induced development of atrophic gastritis but also therapeutically reversed pre-existing precancerous lesions. This effect was accompanied by reduced numbers of proliferating (hyperplastic) cells in infected mice and resulted in restored mucosal architecture. We further provide evidence that PGE_2 mediates its effect on $CD4^+$ T-cells by the transcriptional suppression of IL-2 and its receptor IL-2Rα. IL-2Rα is expressed upon initial encounter of T cells with a specific antigen resulting in IL-2 expression and later upregulation of IL-2Rβ and γ, forming the high-affinity trimeric IL-2R. Activated T cells proliferate rapidly upon IL-2 stimulation, which also acts as a survival factor. Thus, removal of IL-2 leads to T cell anergy and cell death.[296] This notion not only explains the prophylactic effect of PGE_2 treatment but also the therapeutic effect we observe with PGE_2.

We further confirmed this hypothesis by co-administering PGE_2 with recombinant IL-2 (rIL-2) during the infection. Indeed, rIL-2 neutralized the beneficial effects of PGE_2 on the development of *Helicobacter*-associated epithelial atrophy, further underlining the importance of IL-2-driven pathogenic T cell responses in our model. Interesting to note in this context is the fact that the immunosuppressive effect of PGE_2 on $CD4^+$ T-cells was maintained even after explantation from *in vivo* treated mice. We found that $CD4^+$ T-cells from *in vivo* PGE_2 treated mice proliferated less upon anti-CD3/28 stimulation than T-cells derived from untreated mice. Furthermore, PGE_2 treatment of anti-CD3/28 stimulated $CD4^+$ and $CD8^+$ T-cells completely abolished proliferation and cytokine secretion. These results suggest that PGE_2 treatment interferes with antigen-induced T-cell activation and thereby renders T-cells unresponsive to *Helicobacter* infection.

Our data support a model in which PGE_2 directly inhibits the induction or maintenance of the T-effector mediated immune response towards the infection. Previous reports have suggested that tumor-derived PGE_2 induced the activity of regulatory T-cell (Treg) populations by upregulating *FOXP3,* a Treg-specific transcription factor.[297] Mahic *et al.* showed that *in vitro*-induced Treg themselves produce PGE_2 and are able to suppress effector T-cell functions.[128] We tested this hypothesis but observed only a marginally increased regulatory activity of Tregs. This question was addressed by co-culturing various ratios of Treg and Teffectors in the presence of anti-CD3/28, in a setting in which Tregs were pre-treated with PGE_2. PGE_2-pretreated Treg showed a slightly increased potential to suppress Teffector proliferation and IFN-γ secretion. Based on these results, we exclude a major contribution of PGE_2-activated Treg in our model.

Data obtained in our lab showed earlier that $CD4^+$ IFN-γ^+ T cells are key mediators of *Helicobacter*-associated disease, including MALT lymphoma,[282] neonatal *Helicobacter*-tolerance and of vaccine-induced protection against *H. pylori* challenge infection.[18] Here we further extend this model and provide evidence that PGE_2's protective effect is due to inhibited $CD4^+$IFN-γ^+ mediated responses, indirectly by inhibition of the IL-2 feed-back loop. PGE_2 treatment of infected mice showed significantly lower proportions of $CD4^+$ T-cells and *IFNG* gene expression in the gastric mucosa as well as lower numbers of $CD4^+IFN\gamma^+$ cells in the mesenteric lymph nodes, the site of T cell priming.

Taken together we propose that PGE_2-mediated repression of pathogenic T-cells in *Helicobacter*-associated disease may reinforce conventional ET-induced therapy and could show beneficial effects in patients with established intestinal metaplasia.

4.3 Inhibition of Poly(ADP-ribosyl)ation Inhibits and Sustainably Cures *Helicobacter*-Associated Gastric Preneoplastic Lesions

Poly(ADP-ribosyl)ation (PARylation) of PARP1 and other acceptor molecules represents a powerful posttranscriptional modification involved in a variety of physiological and pathophysiological conditions including inflammation and possibly also cancer development (reviewed in Hassa, 2006).[6] Inhibitors of ADP-ribosylation have shown disease-relieving effects in a multitude of chronic inflammation-associated diseases, but have also been effective in rendering *BRCA1*- and *BRCA2*-deficient breast cancer cells susceptible to accumulation of genomic instability, cell cycle arrest and apoptosis.[225]

In this study we provide strong evidence that inhibition of PARylation with the NAD^+ analogue PJ34 suppresses and even cures *Helicobacter*-induced gastritis and epithelial pathology. We show that PJ34 accomplishes this beneficial effect by targeting pathogenic Th1 polarized immune responses. PJ34 treated mice show significantly reduced numbers of stomach-infiltrating neutrophils and pathogenic $CD4^+$ T-cells. This effect was accompanied by suppressed *IFNG* gene transcription in the stomach mucosa. Furthermore, T-cell priming was suppressed as indicated by reduced numbers of $CD4^+IFN\text{-}\gamma^+$ T-cells in the mesenteric lymph nodes of PJ34-treated compared to untreated mice.

Our data are in line with previous studies reporting beneficial effects of PARylation inhibitors in models of chronic inflammation such as inflammatory bowel disease,[209] allergen-induced airway inflammation[198] and endotoxin-induced septic shock.[210] Beneficial effects correlated with defective NF-κB induction, suppressed transcription and activation of IL-6, ICAM-1, E-selectin, TNF-α and IFN-γ mediated responses,[212-214, 298, 299] overall resulting in defective immune responses to the respective antigens.

The beneficial effect of PJ34 treatment in *Helicobacter*-associated disease, which is mainly driven by pathogenic Th1 responses, is illustrated by the following observations: we show that anti-CD3/28 activated $CD4^+$ T-cells produce large amounts of PAR and that this induction is significantly suppressed by PJ34 treatment. The involvement of PAR synthesis in chromatin replication, by modification of histones is a widely accepted concept.[300] Recent data investigating the function of PAR in T-cells suggest that PARylation modifies and positively regulates nuclear factor of activated T-cell (NFAT)-dependent gene transcription.[202, 203] NFAT exerts a critical function in regulating IL-2 transcription.[301, 302] Our data are in agreement with this concept, since PJ34 treatment of anti-CD3/28 stimulated cells lead to drastically suppressed *il-2* (and *IFNG*) gene transcription and a subsequent block of $CD4^+$ and $CD8^+$ proliferation and activation. This observation was further confirmed with the alternative PARP inhibitor, DAM-TIQ-A.

PAR synthesis in T cells seems to be independent of PARP1 or PARP2 (but rather depends on PARP1 AND PARP2), since antiCD3/28 stimulation induced PARylation also in

PARP1$^{-/-}$ and PARP2$^{-/-}$ T-cells. This observation points towards a redundant function of PARP1 and 2 for PAR formation in T cells. Due to embryonic lethality of PARP1/2 double-knock out animals, we could not assess if either PARP reconstitutes PARylation in PARP1- or 2-deficient T-cells. The redundant functions of PARP1 and 2 proteins in catalyzing PARylation provides a possible explanation for why we did not find any difference in infected IL-10$^{-/-}$-deficient compared to IL-10$^{-/-}$/PARP1$^{-/-}$ mice regarding gastritis, epithelial atrophy and intestinal metaplasia at 5 to 21 days post infection. However, it remains to be clarified if PJ34 treatment is efficient also in a PARP1 or 2-deficient genetic background.

In addition to blocking CD4^{+} T-cell migration to the stomach we further show evidence that PJ34 additionally interferes with the T-cell priming in the mesenteric lymph nodes, since PJ34 treatment blocked IFN-γ secretion by MLN cells. Altoghether our data indicate that PJ34 treatment suppresses the *Helicobacter*-specific immune response in the stomach and thus prevents epithelial immunopathology and facilitates bacterial colonization.

Interestingly, we found that PJ34 treatment not only suppresses *Helicobacter*-induced epithelial immunopathology but also successfully cures severe precancerous lesions. However, sustainable restoration of the gastric mucosa was only achieved by combination with bacterial eradication therapy (ET). PJ34 treatment of mice with pre-existing lesions resulted in a significant regression of metaplastic lesions. In contrast to the human situation, ET in mice leads to a significant regression of disease symptoms, though only if administered at early time points after infection[303] (this study). Extended meta-analyses evaluating the long-term benefit of ET in humans concluded that ET had a favourable effect only on gastric atrophy, but did not prevent re-occurrence of metaplastic lesions nor cancer progression,[262, 271, 272] emphasizing the need for treatment options other than sole ET.

The majority of PARylation inhibitors have been designed to inhibit the activity of Poly(ADP-ribose)polymerases (PARPs) by mimicking its substrate NAD^{+},[304] and competing for binding to the catalytic domain.[162, 305] Due to the ubiquitous usage of NAD^{+} as coenzyme, NAD^{+} analogues may target other NAD^{+} dependent enzyme classes as well. Another major concern is that the long-term effects of PARP inhibitiors have not yet been assessed, but require careful consideration.

In summary, we provide evidence that inhibition of PARylation by PJ34 holds promise in preventing and treating *Helicobacter*-associated gastric cancer precursor lesions.

4.4 Therapeutic Implication of PGE_2 and PJ34 Treatments in Related Disease Entities

The above presented results show unambiguously that inhibitors of pathogenic Th1 responses represented by $CD4^+IFN\text{-}\gamma^+$ cells prevent and even resolve *Helicobacter*-induced immunopathology in the stomachs of infected mice.

PGE_2 and PJ34 target effector T-cell populations and render them inert to external stimuli, thereby blocking their proliferation, migration to the site of infection and secretion of effector cytokines. Of the two compounds, PGE_2 showed more pronounced effects in all readouts and seems to have a long-lasting suppressive effect that is sustained also after explantation and *in vitro* re-stimulation. While *in vivo* PGE_2 treatment completely abolished anti-CD3/28-induced IL-2R expression on T-cells, PJ34 treated cells only showed a partial impairment of IL-2R up-regulation and thus retained residual stimulation potential.

Furthermore, addition of rIL-2 to treated $CD4^+$T-cell cultures only rescued the suppressive effect of PGE_2, suggesting that PJ34 regulates T-cell proliferation also by other means. Interestingly, PGE_2 and PJ34 exert similarly suppressive and enduring effects on *IFNG* and *Il-2* transcription that were already observable after a two hour treatment.

The general observation that both PGE_2 and PJ34 possess T-cell suppressing characteristics is not only interesting for *Helicobacter*-associated diseases, but may also have relevance for related T-cell driven diseases. First of all, PGE_2 and PJ34 might be beneficial also in T-cell dependent B-cell tumors such as in MALT lymphoma. A study performed in our laboratory revealed that $CD4^+$T-cells significantly contribute to tumorigenesis, since $CD4^+$T-cell depletion in BALB/c mice by an anti-CD4 antibody resulted in tumor regression.[282] Therefore, inhibition of $CD4^+$T-cell functions using a pharmacological approach may result in the same outcome and might even circumvent adverse effects of immunotherapies. Furthermore, targeting and neutralizing the activation of T cells may also have beneficial effects in other T-cell driven diseases such as T-cell lymphomas and inflammatory skin diseases such as psoriasis.[306] Other wide-spread diseases with as yet limited treatment options and pathogenic T-cell involvement include specific autoimmune diseases such as multiple sclerosis (triggered by T-cell responses against myelin in the central nervous system), diabetes (destruction of insulin-producing β-cells of the pancreas) and others. Another possible indication for PGE_2 or PJ34 treatment in the GI tract would be Crohn's disease, a risk factor for colon carcinogenesis.[296] Interestingly, PARP inhibitors and genetic models have already been tested in models of IBD and have shown encouraging results.[209, 307] Based on our findings, PGE_2 treatment holds great potential to be at least as effective as previously used PARP inhibitors since it resulted in even more drastic relief of disease symptoms in our model. However, PGE_2 as well as PJ34 are robust immunosuppressive compounds and adverse effect may be expected with regard to

enhanced tumor immune escape and enhanced susceptibility to severe infections and their complications.

4.5 *Helicobacter pylori* causes potentially genotoxic DNA double strand breaks in infected cells

Carcinogen-induced DNA damage constitutes an important driving force of carcinogenesis by causing alterations in tumor suppressor genes and oncogenes. Mutations frequently observed in gastric cancers comprise the *TP53* gene at relatively early stages of infection, whereas mutations in *RAS* and 'deleted in colorectal cancer' (DCC) are more commonly reported in metaplastic, dysplastic and cancerous lesions.[7]

We show in this study that *H. pylori* infection efficiently causes DNA double strand breaks (DSB) in primary gastric epithelial cells and various cancer cell lines. DSB breaks are induced in an infection-dose dependent manner and require contact of host cells and live *H. pylori*. We further show that this feature is *Helicobacter*-specific, since *E.coli* DH10B infection does not cause DSB, even at multiplicities of infection (MOI) corresponding to roughly 10000. Interestingly, infected cells are able to repair the *H. pylori*-induced DSB breaks, yet only upon bacterial killing with metronidazole, tetracycline hydrochloride, bismuth subcitrate. This result implies that *H. pylori* continuously assaults the eukaryotic DNA. On the one end, his hypothesis is supported by the persistence of eGFP-MDC1 foci in the very same cell for at least 15 hours, but also by the fact that DNA fragmentation is still observed 54 hours post infection.

Another important point to consider is the *Helicobacte*r-induced inhibition of cell proliferation *in vitro*. So far we have no indication that protein synthesis of DNA repair genes is inhibited as well. Looking at the kinetics, however, it is conspicuous that DNA fragments are observed as early as 1-3 hours post infection. In contrast, PARP activation, generally considered a very early event upon single strand break (SSB) induction,[308] is not induced until 6 hours post *H. pylori* infection. This delayed response to DNA damage may be explained by the absence of single strand breaks (SSB) in *H. pylori*-infected cells or by the not yet initiated DSB repair (which includes SSB formation prior to repair). Another rather speculative possibility includes *H. pylori*-mediated inhibition of the DNA damage repair process downstream of 53BP1 and MDC1.

The rapid induction of DSB as observed by PFGE suggests host-independent induction of DNA damage. Several studies have reported the existence of bacterial genotoxic factors. For example, enteropathogenic bacteria encode a variety of toxins that specifically interfere with eukaryotic host cell signalling and thus possess putative genotoxic potential (genotoxin). Pathogenic and commensal *E.coli* have been reported to express a peptide–polyketide compound, termed colibactin, which causes DBS and subsequent ATM/H2AX activation.[248] The cytolethal distending toxin (CDT) is another potential genotoxin found in *Campylobacter jejuni* and *Helicobacter hepaticus,*[309] that is able to induce DSB in

mammalian cells by the DNase activity of a CDT subunit (CdtB).[310] In murine *H. hepaticus* infection models, CDT is required for colonization,[311] causes chronic intestinal inflammation,[312] and promotes hepatocellular dysplasia.[313] However, there is no evidence for CDT-dependent DSB induction in *H. hepaticus* infection models. Moreover, sequence comparisons reveal that *H. pylori* does not encode a CDT-like protein. We suspect that *H. pylori* derived nucleases may assume the role of the CDT's DNase activity and thereby damage host DNA.

To test the possible involvement of bacterial factors in DNA fragmentation, we plan to perform a comprehensive screen by testing transposon-generated bacterial mutants.[314] The efficiency of the mutants to trigger DNA damage signaling, using a eGPF-MDC1 cell line, will serve as readout and be quantified by light microscopy. The mutant library comprises 5363 independent transposon mutants. 344 of 1500 genes could not be mutated; thus the genome coverage is roughly 3-fold.[315] This approach will enable us to test in parallel a large number of mutants for their ability to induce DSB in the host cell.

Another question that has not been resolved so far is the importance of *H. pylori* DSB *in vivo.* So far, we do not have strong evidence for *H. pylori*-induced DSB in the stomachs of infected mice bearing various kinds of cancer precursors. Bartkova *et al.* reported that ATM-dependent pathways are activated in certain types of cancer precursor lesions (of the urinary bladder, breast, lung and colon) which may delay or even prevent cancer development.[255] However, these authors did not provide data for gastric cancer precursors. A recent study revealed, however, that γH2AX staining was not significantly associated with the Lauren's classification, depth of invasion, lymph node metastasis or TNM stage in patients exposed to atomic-bomb derived radiation.[316] Taken these reports and our data together, it seems that *Helicobacter* infection does not cause massive amounts of DSB *in vivo.* This observation is not thoroughly surprising taken the fact that *Helicobacter*-associated carcinogenesis develops over decades,[317] suggesting that the mutagenic events are relatively infrequent. In addition it should be considered that the MOIs used in our study exceed the number of bacteria present in the stomach; infections with low MOI indeed only caused insignificant amounts of breaks and DSB-specific responses in the cell.

To further clarify the role of damaged DNA in *Helicobacter*-associated disease and its progression we have collected a series of human biopsies in collaboration with Dr.med. S. Cogliatti of the Institute of Pathology, Cantonal Hospital of Skt. Gallen. The selected human biopsies display different grades of *Helicobacter*-associated disease, ranging from superficial gastritis to adenocarcinoma. This sample collection will serve as a valuable tool to monitor changes in the host DNA integrity during the progression of *Helicobacter*-associated gastric disease.

A few reports have investigated the correlations between *Helicobacter* infection, DNA integrity and DNA damage response signalling in order to explain the development of gastric cancer. There is evidence for *Helicobacter*-induced down regulation of proteins involved in mismatch repair (MMR) processes, such as MLH1, PMS1, PMS2, MSH2 and MSH6.[235, 236] Other reports investigated the presence of 8-oxoG in cancerous tissues, a marker of oxidized DNA and correlate of increased DNA damage.[238, 245] These studies suggest that a direct contribution of oxidative damage (derived from ROS generated by infiltrating immune cells) and deficiency of DNA repair account for gastric carcinogenesis. However, there is no profound study addressing the direct contribution of the bacteria to carcinogenesis.

Another interesting preliminary observation is that the degree of epithelial atrophy may be reflected even after explantation. To test this notion, primary gastric epithelial cells were explanted from wild-type mice bearing different grades of epithelial pathology. Interestingly, cells derived from mice with predominantly hyperplastic lesions showed slightly increased numbers of 53BP1 foci upon explantation if compared to cells derived from uninfected mice or mice only showing signs of minor gastritis. This result may reflect the fact that hyperplastic cells activate DSB signalling pathways *in vivo*.

In conclusion, our data provide evidence that *Helicobacter* infection reversibly causes severe DNA lesions in the infected host cells *in vitro* and that this feature may provide a mechanism of carcinogenesis attributed to these stomach-colonizing bacteria.

4.6 Conclusion

The studies presented in this thesis provide experimental proof that the development of *Helicobacter*-associated gastric pre-cancerous lesions can be prevented and pre-existing lesions can be cured by the administration of PGE_2 and PJ34. The beneficial effect of PGE_2 and PJ34 treatments is achieved by the inhibition of $CD4^+IFN\text{-}\gamma^+$ effector T-cell functions. This T-cell population is essential for the development of *Helicobacter*-associated disease and is thus also termed ‘pathogenic’. We show that PGE_2 and PJ34 treatments lead to suppressed $CD4^+$ T-cell proliferation and migration to the infected gastric mucosa, which is accompanied by impaired secretion of T-cell signature cytokines. The therapeutic efficacy of PGE_2 and PJ34 is achieved by abolished IL-2 expression and the subsequent lack of T-cell-dependent immune responses. These findings offer important insights for the development of future therapeutics for patients refractory to antibiotic eradication therapy and patients bearing incurable stages of gastric cancer precursor lesions. Furthermore, we also show that living *Helicobacter* bacteria *per se* damage the host cell DNA in various cell culture models *in vitro*. This observation suggests an intrinsic genotoxic potential of *Helicobacter pylori* that may contribute to gastric cancer development in infected individuals.

5. Abbreviations

53BP1	p53 binding protein 1
ADP	Adenosine diphosphate
AID	activation-induced deaminase
AIF	apoptosis inducing factor
ATM	Ataxia telangiectasia mutated
bp	base pair
BRCA1	breast cancer type I susceptibility factor
CFSE	carboxyfluorescein succinimidylester;
COX	cyclooxygenase
DNA	desoxyribonucleic acid
DSB	DNA double strand break
H2AX	H2A histone
IFN	interferon
IL	interleukin
IMPGE	immortalized primary gastric epithelial cells
IP	intraperitoneal
LPS	Lipopolysachharide
MDC1	Mediator of DNA damage checkpoint protein
MLN	mesenteric lymph node
MOI	multiplicity of infection
NAD	nicotinamide adenine dinucleotide
NFAT	nuclear factor of activated T-cells
NF-KB	nuclear factor-kappa B
PAI	pathogenicity island
PARG	Poly(ADP-ribose)glycohydrolase
PFGE	Pulse Field Gel Electrophoresis
PGE_2	prostaglandin E_2
PJ34	N-(6-Oxo-5,6-dihydro-phenanthridin-2-yl)-N,N-dimethylacetamide
TGF	tumor growth factor
Th1	T-helper 1 cell
TNF	tumor necrosis factor
Teffector	effector T cell ($CD4^+CD25^-$)
Treg	regulatory T cell ($CD4^+CD25^+$)
γH2AX	phosphorylated H2AX

6. References

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7. Curriculum Vitae

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Education

Nov. 2006 – present	**PhD Thesis** Institute of Molecular Cancer Research, University of Zurich, laboratory of Prof. Anne Müller. Topic: Inflammation and Cancer: Targeting pathogenic T-cells inhibits and cures *Helicobacter* -induced gastric preneoplasia in a mouse model.
March – November 2006	**Internship** at Novartis Pharma, Department of Oncology, laboratory of Dr. Thomas Radimerski. Topic: Validation of JAK/AMPK kinases as targets for cancer therapy.
November 2005	**Master of Science** (M.Sc.) in Molecular Biology, Biozentrum, University of Basel.
October 2004 – November 2005	**Master Thesis** in Molecular Microbiology in the laboratory of Prof. Christoph Dehio, Biozentrum, Department of Microbiology, University of Basel. Topic: Translational regulation of a *Bartonella* Type IV secretion system.
October 2004	**Bachelor of Science** in Molecular Biology. Major Subject: Molecular Biology with Cell Biology, Immunology, Biochemistry, Microbiology, Structural Biology and Biophysics. Minor Subject: Medical Biology.
2001-2005	Studies of Molecular Biology at the University of Basel.
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8. Publications

Toller IM, Hitzler I, Sayi A and Mueller A.(2010). Prostaglandin E2 Prevents Helicobacter-Induced Gastric Preneoplasia and Facilitates Persistent Infection in a Mouse Model. *Gastroenterology*.138:1455–1467.

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Toller IM, Neelsen K, Steger M, Altmeyer A, Stucki M, Sartori A, Hottiger MO, Lopes M and Müller A.(2010). *Helicobacter pylori* infection causes potentially genotoxic DNA double strand breaks and triggers DNA damage signalling in eukaryotic host cells. *Manuscript in preparation.*

9. Acknowledgements

First of all I would like to thank Prof. Dr. Anne Müller. Already at our first encounter I knew that I liked the atmosphere here in lab K76 and this has never changed in the last four years. Anne, I would like to thank you for the tremendous support, your motivating way of supervising all of us but also for letting us the freedom to develop our own ideas and to assume responsibility for our projects.

I would also like to acknowledge my thesis committee members: Prof. Dr. Dirk Bumann, Prof. Dr. Christoph Dehio, Prof. Dr. Michael O. Hottiger and Prof. Dr. Josef Jiricny for the critical discussions and helpful suggestions during our meetings.

I am grateful to all collaborators for their invaluable contributions and the prosperous working atmosphere: Dr. Matthias Altmeyer and Prof. Dr. Michael O. Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich) for collaborating on the PARP project, Dr. Kai Neelsen and Prof. Dr. Massimo Lopes, Martin Steger and Prof. Dr. Alessandro Sartori (Institute of Molecular Cancer Research, University of Zürich) and Dr. Stefanie Jungmichel und PD. Dr. Manuel Stucki (Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich) for sharing their expertise in the DNA damage project. Further I would like to thank all other members in the institute for sharing their expert knowledge and reagents, in particular PD Dr. Stefano Ferrari, Payal Bhatia and Wassim Eid, Dr. Barbara Schöpf, Daniela Hühn and Boris Mihaljevic. Further, I would also like to thank our administrative staff and Farah and Malika for their great contributions to our daily life. Also, I would like to thank Daniel Pochetti for his kindness and tremendous help with our mice. A big thank you goes also to Isabelle and Kai for taking the time to read the manuscript, giving me suggestions and for participating in critical and fruitful discussions. Adrian, thanks for putting my findings into medical and understandable German words! Vanessa, my dictionary, I would like to thank you for your patience with correcting all my texts and for adding the Australian flavour to our lab. I would also like to acknowledge the Cancer Biology PhD Program of the Life Science Graduate School for providing such a thorough educational program and useful network.

Another great experience was to work with the people in the Müller Lab. Girls and Mats, thank you all for every single moment, for creating a great scientific environment but also for the fun moments we shared in our leisure (and work) time. I would especially like to thank you, Isabelle, for being my companion on this journey – it was of great support to have you next to me!

Last, I would like to express my great gratitude to my family, Marianne, Marco and Gianina Toller, for their uncompromising support and encouragement and for creating a wonderful home. Adrian, thank you for being at my side and for pursuing our common dream!

10. Appendix

TLR2-activated B-cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing Tr1 cells *(manuscript submitted)*

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Journal: Journal of Immunology

Contribution: Experimental assistance for adoptive transfer experiments, discussion of the manuscript

Summary: This manuscript provides evidence that excessive Helicobacter-induced Th-1 driven gastric immunopathology is restricted by cooperating 'regulatory' B- and T-cells. Using mouse strains with defined defects in either of the two populations, we found that B-cells, upon their MyD88-dependent activation by *Helicobacter* TLR-2 ligands, are able to efficiently induce IL-10-dependent suppressive activity in conventional $CD4^+$ T-cells, converting these into T regulatory-1 (Tr-1)-like cells. The cooperative activity of 'regulatory' B-cells and Tr-1 cells prevents the development of gastric premalignant lesions, thus providing evidence that B-cells can have important immunomodulatory function during immune responses to persistent bacterial infections.

TLR-2-activated B-cells suppress *Helicobacter*-induced preneoplastic gastric immunopathology by inducing T regulatory-1 cells[1]

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Running title: B-cell-induced Tr-1 cells prevent preneoplasia

Key words: T-cells, inflammation, bacterial infection, Breg, T-regulatory-1

Abstract

B-cells regulate autoimmune pathologies and chronic inflammatory conditions such as autoimmune encephalomyelitis and inflammatory bowel disease. The potential counter-regulatory role of B-cells in balancing pathogen-specific immune responses and excessive immunopathology is much less understood due to the lack of appropriate persistent infection models. We show here that B-cells have the ability to negatively regulate adaptive immune responses to bacterial pathogens. Using mouse models of infection with *Helicobacter felis,* a close relative of the human gastrointestinal pathogen *H. pylori*, we found that B-cells activated by *Helicobacter* TLR-2 ligands induce IL-10-producing $CD4^+CD25^+$ T regulatory-1 (Tr-1)-like cells *in vitro* and *in vivo*. Tr-1 conversion depends on TCR signalling and a direct T-/B-interaction through CD40/CD40L and CD80/CD28. B-cell-induced Tr-1 cells acquire suppressive activity *in vitro* and suppress excessive gastric *Helicobacter*-associated immunopathology *in vivo*. Adoptive co-transfer of MyD88-proficient B-cells and Tr-1 cells restores a normal gastric mucosal architecture in $MyD88^{-/-}$ and $IL\text{-}10^{-/-}$ mice in a manner that depends on T-cellular, but not B-cellular IL-10 production. Our findings describe a novel mechanism of B-cell dependent Tr-1 cell generation and function in a clinically relevant disease model. In conclusion, we demonstrate here that the B-cell/Tr-1 cell axis is essential for balancing the control of *Helicobacter* infection with the prevention of excessive Th1-driven gastric immunopathology, promoting gastric mucosal homeostasis on the one hand and facilitating *Helicobacter* persistence on the other.

Introduction

B-cells have emerged in recent years as important regulators of T-cell-driven autoimmune pathologies and of excessive immune responses to the indigenous microbiota (1-4). The first evidence for a regulatory role of B-cells was described in mouse models of experimental autoimmune encephalomyelitis (EAE) (5). More recently, B-cells were shown to also suppress excessive inflammation in models of collagen-induced arthritis (4, 6), type I diabetes (7), contact hypersensitivity (8) and inflammatory bowel disease (9, 10). The proposed mechanisms of B-cell-driven immunoregulation include the production of IL-10 (9, 11, 12) and the activation of $CD4^+CD25^+$ or $CD8a^+$ T regulatory cells (Treg) via a direct interaction (10, 13).

In contrast to Treg, which represent phenotypically and developmentally distinct lineages, B-cells with regulatory properties (sometimes termed 'Breg') remain ill defined with respect to their immunophenotype, preferred anatomical location and ontogeny. All B-cells can in principle produce IL-10 upon TLR-2, -4 or -9 stimulation or T-cell-dependent activation (14, 15), with particularly strong production being attributed to $CD5^+$ peritoneal B-1a cells (16), intestinal $CD19^+CD1d^{hi}$ cells (9) and splenic $CD1d^{hi}CD5^+$ 'B10' cells (8). Other B-cell subsets with reported IL-10-dependent regulatory activity include splenic $CD21^{hi}CD23^+IgM^+$ transitional 2 marginal zone precursor cells (6) and $CD21^{hi}CD23^{lo}$ marginal zone B-cells (10).

Little information is available regarding 'regulatory' B-cell activity in bacterial infectious diseases, possibly due to the fact that most bacterial infections take an acute rather than chronic course in humans and the respective experimental models (17). Exceptions include *Mycobacterium tuberculosis* and the widespread bacterial pathogen *Helicobacter pylori*, a gram-negative colonizer of the human gastric mucosa (17). *Helicobacter* infections are acquired during childhood and, despite triggering strong local and systemic immune responses, typically persist for life. Whereas a majority of infected individuals remain asymptomatic, ~20% develop one or more *Helicobacter*-associated severe gastric and duodenal disease manifestations; these include chronic active gastritis, ulcers, gastric B-cell lymphoma and, rarely, gastric adenocarcinoma (18-21). While the bacterial virulence factors, host genetic traits and environmental parameters predisposing to *Helicobacter*-associated disease have been the subject of intense investigation, much less is known about the protective mechanisms that operate in the majority of infected individuals not developing disease symptoms (22-26).

We have reported recently using a mouse model of *Helicobacter felis*-induced gastric preneoplasia that C57BL/6 mice recapitulate the human scenario, as even closely related animals differ substantially with respect to disease susceptibility (27). A subset of infected mice displayed the histopathological changes indicative of early gastric carcinogenesis, i.e. the appearance of a chronic inflammatory infiltrate, the loss of specialized gastric lineages (a

process termed 'atrophy' or 'atrophic gastritis'), the compensatory hyperproliferation of undifferentiated progenitor cells ('hyperplasia') and the development of intestinal metaplasia. In contrast, their less susceptible littermates were largely protected from the development of such lesions (27). We further showed that Th-1-polarized T-cells and their signature cytokine IFN-γ cause preneoplastic gastric transformation in susceptible animals, and that these cells are essential for control of the infection (27). Additional evidence for this model came from our recent observation that the pharmacological targeting of Th-1 cells efficiently prevents and even reverts preneoplastic gastric pathology (28).

Here we show that excessive *Helicobacter*-induced Th-1 driven gastric immunopathology is restricted by cooperating 'regulatory' B- and T-cells. Using mouse strains with defined defects in either of the two populations, we found that B-cells, upon their MyD88-dependent activation by *Helicobacter* TLR-2 ligands, are able to efficiently induce IL-10-dependent suppressive activity in conventional $CD4^+$ T-cells, converting these into T regulatory-1 (Tr-1)-like cells. The cooperative activity of 'regulatory' B-cells and Tr-1 cells prevents the development of gastric premalignant lesions, thus providing evidence that B-cells can have important immunomodulatory function during immune responses to persistent bacterial infections.

Materials and Methods

Animal experimentation

C57BL/6, TCR-$\beta^{-/-}$BL/6, Rag-$1^{-/-}$BL/6, IL-$10^{-/-}$BL/6, MyD$88^{-/-}$BL/6, TLR-$2^{-/-}$BL/6 and Ly5.1BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). *IL-10*-GFP reporter mice ('TIGER') were described previously (29). Mice with a B-cell-specific or $CD4^+$/$CD8^+$ T-cell-specific inactivation of *IL10* were generated by crossing CD19-Cre or CD4-Cre mice with $IL10^{flox/flox}$ mice (30, 31). All mice were bred at a University of Zurich SPF facility and maintained in individually ventilated cages. All animal experiments were approved by the cantonal veterinary office. Mice were infected at 5-6 weeks of age with two consecutive orogastric doses of *H. felis*, which was grown as described (27). For adoptive transfer experiments, immunomagnetically sorted splenic $CD4^+CD25^-$ T-cells from *H. felis* infected donors were injected into the tail veins of recipient mice, either alone or in combination with equal numbers of $CD4^+CD25^+$ Treg. *In vivo* depletion of regulatory T-cells was achieved by weekly i.p. injections of 1x100µg and 3x50µg of anti-CD25 antibody (clone PC-61.5). Recombinant IL-10 (BD Pharmingen) was administered i.p. thrice weekly at 270ng/dose.

Assessment of H. felis *colonization, IFN-γ expression and gastric histopathology*

Stomachs were retrieved and dissected longitudinally into several equally sized pieces. For the qPCR-based assessment of *H. felis* colonization, genomic DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and subjected to qPCR analysis of the *flaB* gene as described (27). For the quantitative analysis of gastric IFN-γ expression, total gastric RNA was subjected to real time RT-PCR using primers and conditions specified previously (27). For the quantitative assessment of gastric histopathology, Giemsa-stained paraffin-embedded stomach sections were scored on a scale of 0-6 for the parameters chronic inflammation, atrophy, epithelial hyperplasia and metaplasia as described in detail previously (27). All pictures were taken with a 20x magnification on a Leica Leitz DM RB microscope equipped with a DFC 420C camera. Images were acquired using the Leica Application Suite 3.3.0 software. Scale bars indicate 50µm.

Cell culture and flow cytometry

Gastric lymphocyte and mesenteric lymph node single cell suspensions were generated by digestion with 0.25mg/ml collagenase IV (Sigma) and passing through a cell strainer. $CD4^+CD25^-$ T-cells and B-cells were purified from single cell suspensions of freshly isolated spleens or mesenteric lymph nodes by immunomagnetic sorting (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. IL-10 secretion was measured by ELISA (BD Biosciences, San Diego, CA, USA). Directly coupled antibodies for the following markers were used for flow cytometry: CD4 (clone RM4-5, eBioscience, San Diego, USA), CD25 (clone PC-61.5, R&D Systems), CD19 (clone 6D5, Abcam, Cambridge, UK), CD80 (clone 16-10A1, Biolegend), CD86 (clone GL1, BP Pharmingen), IgM (polyclonal goat serum, Southern Biotech), CD21 (clone 7G6, BD Pharmingen), CD23 (clone B3B4, Biolegend), CD5 (clone 53-7.3 BD Pharmingen), CD1d (clone 1B1, BD Pharmingen), CD45 (clone 30-F11, Biolegend), B220 (clone RA3-6B2, BD Pharmingen), Ly5.1/CD45.1 (clone A20, Biolegend). All FACS analyses were performed on a CyanADP instrument (Dako, Glostrup, Denmark). FACS sorting was performed on a FACS Aria cell sorter (BD Biosciences). For B-/T-cell co-cultures, immunomagnetically sorted B-cells and $CD4^+CD25^-$ T cells were cultured at a 1:1 ratio in 96 well round bottom plates (Nunc, Roskilde, Denmark) at 2×10^5 cells/well in the presence of 1µg/ml anti-CD3 (clone 145-2C11; Bio X Cell) and 10ng/ml recombinant IL-2 (R&D Systems). Blocking antibodies to CD40L (clone MR1, BD Biosciences) and CTLA-4 (clone 4F10) or agonistic anti-CD28 antibody (clone 37.51, Biolegend) were added where appropriate. Cells were separated by transwell filters as indicated. After four days, cells were harvested, stained and analyzed by flow cytometry or used for adoptive transfer experiments. For *in vitro* suppression assays, $Ly5.1^+CD4^+CD25^-$ T cells

were labelled with 5□M carboxyfluorescein succinimidyl ester (CFSE) and stimulated with anti-CD3/CD28-coated Dynabeads (Invitrogen, Carlsbad, CA, USA) in the presence or absence of equal numbers of FACS-sorted $CD4^+CD25^+$*il-10*-GFP^+ or $CD4^+CD25^+$*il-10*-GFP^- T cells. Cells were harvested after five days and the CFSE signal of $Ly5.1^+$ lymphocytes was analyzed by flow cytometry.

Statistical analysis

All p-values were calculated using Graph Pad prism 5.0 or R software. The significance of categorical differences in histopathology scores was calculated by Mann-Whitney or unpaired Wilcox test and the significance of numerical differences was calculated by Student's t-test. In all graphs showing colonization data and histopathology scores, the medians are indicated by horizontal bars. In column bar graphs, standard errors of the mean are indicated by vertical bars. n.a. denotes 'not applicable', and n.s. stands for 'not significant'.

Results

Regulatory T-cells restrict Helicobacter*-induced gastric preneoplastic immunopathology*

Immunodeficient mouse strains lacking either T-cells (TCR- $\beta^{-/-}$) or T- and B-cells (Rag-$1^{-/-}$) are protected from the gastric histopathology observed in *H. felis*-infected wild type C57BL/6 mice (27). The adoptive transfer of pure populations of splenic $CD4^+CD25^-$ effector T-cells from *H. felis*-infected donors into hosts of both immunodeficient backgrounds causes severe gastric mucosal inflammation and the rapid development of preneoplastic lesions within only four weeks of infection (Figure 1A,B,E,F). The lesions are histologically evident as chronic atrophic gastritis, epithelial hyperplasia and intestinal metaplasia (Figure 1A,B,E,F). The gastric immunopathological response to the infection is accompanied by a strong increase in local IFN-γ levels (Figure 1C,G) and a concomitant reduction in *Helicobacter* burden (Figure 1D,H). The co-transfer of equal numbers of spleen-derived $CD4^+CD25^+$ regulatory T-cells with the effector cells prevented gastric IFN-γ production, *Helicobacter* clearance and the development of gastric lesions in the TCR-$\beta^{-/-}$ background (Figure 1A-D), but not in the Rag-$1^{-/-}$ background (Figure 1E-H). We deduce from this observation that the suppressive activity of co-transferred Treg on $CD4^+$ effector T-cells depends on a cell type that is present in the TCR- $\beta^{-/-}$, but not the Rag-$1^{-/-}$ strain.

To verify the importance of $CD4^+CD25^+$ Treg in controlling excessive gastric immunopathology in *H. felis*-infected wild type mice, we depleted Treg systemically by twice-weekly injections of anti-CD25 antibody during the entire four week time course of infection (supplemental Figure 1). This treatment resulted in the strikingly accelerated development of atrophy and hyperplasia and

a significantly reduced bacterial burden (supplemental Figure 1A-C). In conclusion, the data suggest that Treg effectively limit excessive gastric immunopathology and, at the same time, prevent *Helicobacter* clearance. The failure of Treg to function in the Rag-1$^{-/-}$ background further argues that additional cell types are required for optimal suppressive activity of Treg in this scenario; possible candidate cell types include B-cells.

B-cells produce IL-10 and up-regulate CD80 in a MyD88-dependent manner upon exposure to Helicobacter*-derived TLR-2 ligands*

Hypothesizing that B-cells might have an important role in (co-) regulating *Helicobacter*-specific T-cell responses and gastric immunopathology, we analyzed the response of B-cells to *Helicobacter* sonicate. We examined expression of the activation markers CD80 and CD86 as well as IL-10 production; we focused on this cytokine because IL-10$^{-/-}$ mice develop more severe preneoplastic gastric pathology upon *H. felis* infection than wild type mice infected for the same time frame (supplemental Figure 2). To analyze IL-10 expression at the single cell level, we took advantage of a reporter mouse that was generated by insertion of a GFP element in front of the polyadenylation site of the *il-10* gene (29). Treatment of immunomagnetically purified splenic *il-10*-GFP knock-in B-cells with *Helicobacter* sonicate revealed a robust *il-10*-GFP^{+} population that strongly expressed CD19 (Figure 2A), IgM, CD21 and CD23 (Figure 2B). The CD21^{+}CD23^{+} phenotype is characteristic of the transitional 2 marginal zone precursor cell lineage that was recently reported to be an important source of B-cellular IL-10 (6). The TLR-2 and -4 ligands PAM3Cys and *E. coli* LPS also strongly induced *il-10*-GFP (Figure 2A); this was further confirmed by IL-10 ELISA of the culture supernatants (Figure 2C). A comparison of the responsiveness of B-cells from various anatomical locations and lymphoid organs to *Helicobacter* sonicate revealed that all B-cells, independent of whether they had been isolated from the spleen, peritoneum, mesenteric lymph nodes or Peyer's patches, could be stimulated to express IL-10 upon exposure to *Helicobacter*, albeit at variable levels (supplemental Figure 3).

IL-10 secretion by purified B-cells absolutely required MyD88 signaling and TLR-2 as B-cells isolated from the respective MyD88$^{-/-}$ and TLR-2$^{-/-}$ animals failed to respond to *Helicobacter* extract or PAM3Cys (Figure 2C); in contrast, TLR-4$^{-/-}$ and TLR-9$^{-/-}$ B-cells showed normal IL-10 production upon *Helicobacter* exposure (data not shown). The treatment of B-cells with *Helicobacter* sonicate further strongly induced the surface expression of CD80 (Figure 2D) and CD86 (supplemental Figure 4), and IL-10 production was detected in CD80^{+}, but not CD80^{-} B-cells (Figure 2D). The up-regulation of surface CD80 on B-cells was entirely dependent on MyD88 and TLR-2, as B-cell preparations from the respective k/o animals failed to induce CD80 upon stimulation with *Helicobacter* sonicate (Figure 2E, supplemental Figure 4). In contrast, the

up-regulation of CD86 appeared to be only partially MyD88- and TLR-2-dependent (supplemental Figure 4). Interestingly, $CD80^+$ cells also expressed higher levels of CD40, with mean fluorescence intensities of CD40 being more than twice as high in $CD80^+$ than in $CD80^-$ cells (MFI=70 vs. 156; Figure 2E). In conclusion, the results suggest that B-cells sense *Helicobacter* in a TLR-2- and MyD88-dependent manner, and respond to *Helicobacter*-derived TLR-2 ligands by up-regulating CD40, CD80 and CD86 and by secreting IL-10.

Co-transfer of B-cells and Treg or regular doses of recombinant IL-10 alleviate excessive inflammation and Helicobacter*-associated immunopathology in MyD88$^{-/-}$ mice*

As the B-cell-specific induction of IL-10 and CD40/CD80 expression requires MyD88 signaling, we hypothesized that $MyD88^{-/-}$ mice would show enhanced susceptibility to *Helicobacter* infection. Indeed, $MyD88^{-/-}$ mice displayed strongly accelerated *Helicobacter*-induced gastric histopathology, developing severe atrophy, hyperplasia and metaplasia as early as one month post infection (Figure 3A,B). The phenotype of $MyD88^{-/-}$ mice is therefore reminiscent of $IL\text{-}10^{-/-}$ animals. However, in contrast to $IL\text{-}10^{-/-}$ mice, which consistently clear the infection before one month p.i. (supplemental Figure 2) (32), $MyD88^{-/-}$ mice are colonized as densely as wild type animals at this time point (Figure 3C).

To test whether the elevated susceptibility of $MyD88^{-/-}$ mice is due to their defect in IL-10 production, a group of infected $MyD88^{-/-}$ mice received thrice-weekly intraperitoneal injections of recombinant murine IL-10 for the four week duration of the experiment (Figure 3A-C). Interestingly, the IL-10 treatment significantly reduced the severity of all parameters of gastric histopathology (Figure 3A-C), indicating that IL-10 deficiency is the likely cause of the high susceptibility of $MyD88^{-/-}$ mice to *Helicobacter*-induced gastric preneoplastic changes.

Having identified TLR-2 as the relevant receptor for *Helicobacter*-derived PAMPs in purified B-cells (Figure 2), we asked whether $TLR\text{-}2^{-/-}$ mice would show a similarly enhanced susceptibility to *Helicobacter* infection as $MyD88^{-/-}$ animals. Indeed, $TLR\text{-}2^{-/-}$ mice developed more severe pathology than wild type controls and, in contrast to $MyD88^{-/-}$ mice, were able to reduce the bacterial burden significantly (supplemental Figure 5). $TLR\text{-}4^{-/-}$ and $TLR\text{-}9^{-/-}$ mice did not differ from wild type animals in terms of their colonization levels and gastric histopathology (data not shown), indicating that TLR-2 is the predominant receptor sensing *Helicobacter in vivo*.

In order to assess whether wild type B-cells are able to prevent the accelerated gastric histopathology characteristic of $MyD88^{-/-}$ mice, we adoptively transferred purified splenic B-cells to $MyD88^{-/-}$ mice at the time of experimental infection. No difference in the severity of gastric lesions could be detected between the recipient group and infected $MyD88^{-/-}$ mice that had not received B-cells (Figure 3D,E). Similarly, adoptive transfer of purified splenic $CD4^+CD25^+$ Treg

also failed to prevent gastric pathology (Figure 3D,E). In contrast, the co-transfer of both cell types significantly reduced all parameters of gastric histopathology in recipient mice (Figure 3D,E), suggesting that B-cells and Treg cooperate in the control of excessive immunopathological responses to *Helicobacter*.

Helicobacter-*activated B-cells induce Tr-1-like cells with suppressive activity in a TLR-2, MyD88- and contact-dependent manner*

B-cells respond to *Helicobacter* with the MyD88- and TLR-2-dependent production of IL-10 and up-regulation of CD80 and CD40 (Figure 2); however, adoptively transferred B-cells prevent *Helicobacter*-induced pathology in MyD88$^{-/-}$ animals only in the presence of co-transferred T-cells (Figure 3D,E). We speculated that *Helicobacter*-exposed B-cells might trigger a suppressive phenotype in conventional T-cells and/or enhance the suppressor functions of Treg. To address these possibilities, we co-cultured immunomagnetically purified splenic B- and CD4^{+}CD25^{-} T-cell populations in the presence of crosslinking anti-CD3 antibody and IL-2, with or without prior exposure of the B-cells to *Helicobacter* sonicate, PAM3Cys or LPS. While naïve B-cells were largely incapable of inducing GFP expression in *il-10*-GFP knock-in CD4^{+}CD25^{-} T-cells, a brief pretreatment of the B-cells with either *Helicobacter* sonicate, PAM3Cys or LPS prior to co-culturing efficiently triggered the B-cells' ability to induce *il-10*-GFP expression and IL-10 secretion by T-cells as determined by FACS and ELISA (Figure 4A-C). *Il-10*-GFP^{+} T-cells expressed high levels of CD25 (Figure 4A), and were FoxP3-negative (data not shown), suggesting that the interaction with B-cells had converted conventional T-cells to a T-regulatory-1 (Tr-1)-like phenotype. Interestingly, T-cellular IL-10 expression was dependent on a direct interaction between both cell types, as their physical separation by transwell filters prevented T-cellular *il-10*-GFP and IL-10 expression (Figure 4A-C). Addition of a blocking antibody to CD40L, but not CTLA-4, prevented *il-10*-GFP/IL-10 expression (Figure 4A-C). B-cell-induced *il-10*-GFP/IL-10 production by T-cells depended on MyD88 signaling and TLR-2, but not on IL-10 proficiency on the part of the B-cell (Figure 4D,E). This observation is in line with our finding that the induction of CD40 and CD80 on B-cells by *Helicobacter* sonicate, PAM or LPS depends on MyD88 and TLR-2 (Figure 2E). Interestingly, addition of an agonistic anti-CD28 antibody to the co-cultures strongly enhanced the induction of *il-10*-GFP^{+}CD25^{+} cells, both by naïve and by *Helicobacter*-pretreated B-cells (Figure 4F), indicating that the TLR-2- and MyD88-dependent up-regulation of CD80/CD86 on B-cells provides a co-stimulatory signal for the generation of Tr-1-like cells. In summary, exposure to *Helicobacter* TLR-2 ligands converts B-cells into efficient inducers of IL-10-producing CD25^{+} Tr-1-like cells, and this process depends on a direct

interaction of the two cell types via CD40/CD40L, a TCR stimulus (αCD3) and a co-stimulatory signal (αCD28).

To determine whether B-cell-induced Tr-1-like T-cell populations have acquired suppressive activity, we measured the CFSE dilution of $CD4^+CD25^-Ly5.1^+$ T-cells activated by anti-CD3/CD28 crosslinking in the presence or absence of FACS-sorted $CD4^+CD25^+$*il-10*-GFP^+ and $CD4^+CD25^+$*il-10*-GFP^- populations (Figure 4G). $CD25^+$ *il-10*-GFP^+ T-cells suppressed $Ly5.1^+$ T-cell proliferation more effectively than their $CD25^+$ *il-10*-GFP^- counterparts (Figure 4G), indicating that B-cell-induced Tr-1-like populations have indeed acquired suppressive properties.

Tr-1-like cells are generated in vivo *and recruited to the* Helicobacter*-infected gastric mucosa*

To determine whether Tr-1-like cells and other IL-10-producing immune cells are generated *in vivo* upon infection with *Helicobacter*, we infected *IL-10*-GFP knock-in mice with *H. felis* for three months and analyzed the GFP expression of various hematopoietic lineages in the stomach and in several lymphoid organs -the spleen, MLN and Peyer's patches- in comparison to an uninfected control group. *IL-10*-GFP^+ cells were readily detectable in all organs due to their robust signal intensity. No significant changes in *IL-10*-GFP^+ cell numbers could be detected in the Peyer's patches and spleens of infected vs. uninfected mice with regard to the B-cell-, T-cell, macrophage and DC lineages (using the markers CD4, CD19, F4/80 and CD11c; data not shown). In contrast, stomach and MLN preparations revealed clear infection-associated changes in the *IL-10*-GFP^+ population, especially in the $CD4^+$ T-cell compartment (Figure 5A,B). In the MLN, the fraction of *IL-10*-GFP^+ cells in the $CD4^+$ population increased from on average 3.2 to 6.2% due to infection (Figure 5A); the majority of *IL-10*-GFP^+ cells were CD25-positive (Figure 5A). The overall $CD25^+$ fraction increased as well, from 25% to 35% of the total $CD4^+$ population (Figure 5A, lower panel). In the stomach, the number of infiltrating $CD4^+$ T-cells was on average five times higher in the infected compared to the uninfected mice (Figure 5B); strikingly, the number of $CD4^+$*IL-10*-GFP^+ T-cells increased 13 fold due to infection, suggesting that Tr-1-like cells are either preferentially recruited to the stomach or directly generated there (Figure 5B). Consistent with their Tr-1-like phenotype, most $CD4^+$*IL-10*-GFP^+ cells in the stomach stained positive for CD25 (Figure 5B).

As exposure to *Helicobacter* TLR ligands induces IL-10 expression and CD80 up-regulation on B-cells *in vitro*, we quantified the *IL-10*-GFP^+ and $CD80^+$ subpopulations of $CD19^+$ B-cells. In the MLN, the fraction of *IL-10*-GFP^+ B-cells doubled from 0.7 to 1.5% due to infection (Figure 5C). Consistent with the induction of CD80 and IL-10 expression in B-cells *in vitro* by *Helicobacter* ligands (Figure 2D), all *IL-10*-GFP^+ B-cells in the MLN stained positive for CD80 (data not shown). The overall fraction of $CD80^+$ B-cells was also significantly greater in the MLN of

infected vs. uninfected mice (14 vs. 10%; Figure 6C), indicating that MLN B-cells respond to *Helicobacter* infection in the stomach in a readily detectable manner. In the gastric mucosa, B-cells were six times more abundant in infected compared to uninfected animals (Figure 5D). However, the absolute number of stomach-infiltrating $CD19^+$*IL-10*-GFP^+ cells was comparable in both groups (Figure 5D), indicating that the infected gastric mucosa is targeted preferentially by non-IL-10-expressing B-cells. All stomach-infiltrating *IL-10*-GFP^+ B-cells were $CD5^+CD1d^+$, independent of the infection status of the mouse (data not shown), indicating that they belong to the recently described 'B10' subset of B-cells with regulatory function (8). Interestingly, roughly half of all stomach-infiltrating $CD19^+$ B-cells were CD80-positive, compared to only 8-13% of B-cells in the lymphoid organs analyzed (data not shown). In summary, large numbers of activated $CD80^+$ B-cells infiltrate the stomach in response to *Helicobacter* infection, yet only a minor fraction of these stomach-infiltrating B-cells produce IL-10. Overall, the results obtained in the *IL-10*-GFP knock-in mouse imply that, whereas both *IL-10*-GFP^+ T- and B-cells are generated in the gut-draining lymph nodes as a result of *Helicobacter* infection, only *IL-10*-GFP^+ T-cells are recruited to the infected stomach with high efficiency.

The control of gastric preneoplastic pathology requires IL-10 expression by T-, but not B-cells

B-cells constitute a direct source of IL-10, and also trigger IL-10 production by $CD4^+$ T-cells upon activation by *Helicobacter*-derived TLR-2 ligands both *in vitro* and *in vivo* (Figures 2, 4, 5). To clarify *in vivo* whether either one or both cell types require IL-10 for the efficient suppression of *Helicobacter*-induced gastric immunopathology, we took advantage of mouse strains with a conditional deletion of the IL-10 gene in the T-cell- (31) or B-cell compartments (30). Infection of $IL\text{-}10^{fl/fl}$CD4-Cre mice with *Helicobacter felis* resulted in the significantly accelerated development of gastric pathology compared to $IL\text{-}10^{fl/fl}$ mice (Figure 6A,B). In contrast, the $CD19^+$ B-cell-specific loss of IL-10 did not render the mice more susceptible to *Helicobacter*-induced pathology (Figure 6A,B). Consistent with their rapid development of immunopathological changes, $IL\text{-}10^{fl/fl}$CD4-Cre mice were colonized at significantly lower levels than either $IL\text{-}10^{fl/fl}$ or $IL\text{-}10^{fl/fl}$CD19-Cre mice (Figure 6C) and produced significantly higher levels of gastric IFN-γ (Figure 6D). All three read-outs combined indicate that T-cell-derived IL-10 is essential, but B-cell-derived IL-10 is dispensable, for immune counter-regulation of Th-1-driven immunopathology in our model of *Helicobacter*-associated gastric preneoplasia.

B- and T-cells co-operate in vivo *to suppress* Helicobacter-*induced gastric immunopathology in* $MyD88^{-/-}$ *and* $IL\text{-}10^{-/-}$ *mice*

Having demonstrated that the co-transfer of splenic B-cells and $CD4^+CD25^+$ T-cells into $MyD88^{-/-}$ recipients alleviates the pathology characteristic of this strain (Figure 3D,E), and that B-cell-induced Tr-1-like cells possess suppressive activity *in vitro* (Figure 4G), we asked whether B-cells and B-cell-induced Tr-1 cells would synergize to prevent excessive infection-associated pathology in $MyD88^{-/-}$ and $IL\text{-}10^{-/-}$ mice. To address this question, we adoptively transferred B-/T-cell co-cultures that were grown under conditions leading to Tr-1 conversion (as described in Figure 4A) into recipients of both genetic backgrounds. Interestingly, the transfer of co-cultures consisting of wild type B- and T-cells completely inhibited the accelerated pathology of $MyD88^{-/-}$ mice (Figure 7A,B). IL-10 proficiency of T-cells, but not B-cells, was essential for conferring protection (Figure 7A,B), an observation that is in line with the phenotypes of the respective conditional $IL\text{-}10^{-/-}$ mice (Figure 6). A total of 250.000 transferred cells were sufficient to prevent gastric pathology in $MyD88^{-/-}$ mice; the transfer of pure B-cell or T-cell populations did not alter the severity of the lesions (data not shown).

Finally, we utilized $IL\text{-}10^{-/-}$ recipients to test whether MyD88 proficiency is required on the part of the B-cells for prevention of the gastric pathology characteristic of this strain. Interestingly, protection was conferred in this background only by co-cultures of wild type T- and B-cells (Figure 7C,D); $MyD88^{-/-}$ B-cells, which are incapable of inducing Tr-1 cells upon exposure to *Helicobacter* (Figure 4D,E), did not alleviate the gastric pathology of $IL\text{-}10^{-/-}$ recipients if co-transferred with WT T-cells (Figure 7C,D). The clearance of *Helicobacter* typically observed in $IL\text{-}10^{-/-}$ mice was prevented in the recipients of WT B-cell/WT T-cell co-cultures, but not the recipients of $MyD88^{-/-}$ B-cell/WT T-cell co-cultures (Figure 7E). Finally, the induction of gastric IFN-γ which typically accompanies clearance (Figures 1, 6), was suppressed by the transfer of WT B-cell/WT T-cell co-cultures, but not $MyD88^{-/-}$ B-cell/WT T-cell co-cultures (Figure 7F). In summary, we conclude that B-cells and B-cell-induced Tr-1 cells synergize to prevent excessive *Helicobacter*-associated inflammation and immunopathology *in vivo*, and that B-cells require MyD88 signaling for co-operative counter-immune regulation.

Discussion

We show here using mouse models of *Helicobacter*-induced gastric premalignant pathology that B-cells have an important role in fine-tuning the adaptive Th-1-driven immune response to the infection and in thereby limiting excessive gastric immunopathology. This regulatory function of B-cells depends on their ability to sense and respond to *Helicobacter* via TLR-2 and MyD88 and to directly interact with naïve T-cells, which are efficiently converted to Tr-1-like, $CD4^+CD25^+IL\text{-}10^+$ cells in a CD40L and CD28-dependent manner. B-cell-induced Tr-1 cells acquire suppressive activity *in vitro* and -in collaboration with B-cells- block gastric histopathological

changes *in vivo* in genetic backgrounds that are particularly susceptible to *Helicobacter*-associated pathology. Our findings provide a plausible mechanistic explanation for epidemiological and experimental data showing that the majority of chronically *Helicobacter*-infected individuals and experimentally infected animals remain asymptomatic despite being colonized with virulent *H. pylori* strains and despite harboring high bacterial loads (27, 33, 34).

B-cells have been shown to possess regulatory activity in several mouse models of chronic inflammatory conditions or autoimmune diseases, including models of multiple sclerosis (EAE) (5, 11, 13, 15, 35), collagen-induced arthritis (6, 12), contact hypersensitivity (8), type I diabetes (7) and inflammatory bowel disease (9, 10). The prevailing mechanistic model of B-cell immune counter-regulation postulates that specific subsets of IL-10-producing B-cells possess regulatory function. Several studies have shown a strict dependence of B-cell regulatory activity on IL-10 production by the B-cells themselves (6-8, 11, 12, 30, 35); other proposed mechanisms of B-cell-mediated immune regulation include the contact-dependent induction or activation of $CD4^+CD25^+$ or $CD8a^+$ Treg (10, 13, 36). We show here that, although exposure to *Helicobacter*-derived TLR-2 ligands induces IL-10 production in B-cells in a MyD88-dependent manner, IL-10 is not required on the part of the B-cell for suppression of *Helicobacter*-induced pathology. Rather, *Helicobacter*-activated B-cells acquire the ability to efficiently induce Tr-1-like cells, an abundant population of intraepithelial lymphocytes in the small intestine known to suppress excessive intestinal inflammation (29). Therefore, while IL-10 induction in the B-cells coincides with their TLR-2- and MyD88-dependent activation and their acquisition of T-cell-converting capabilities, the cytokine itself is clearly not required for Tr-1 cell generation. Evidence that IL-10 production by Tr-1 cells, but not B-cells, is crucial for protection from *Helicobacter*-induced preneoplasia came from the analysis of mouse strains with either a T-cell- or B-cell-specific ablation of the *il-10* gene. $IL\text{-}10^{fl/fl}$CD4-Cre animals displayed significantly accelerated gastric pathology upon infection compared to wild type mice; in fact, their phenotype was indistinguishable from the phenotype of a complete $IL\text{-}10^{-/-}$ strain. In contrast, $IL\text{-}10^{fl/fl}$CD19-Cre mice did not exhibit increased susceptibility to gastric immunopathology. In line with these findings, only IL-10-producing T-cells, but not IL-10-producing B-cells, were found to be recruited to the gastric mucosa upon infection, indicating that $IL\text{-}10^+$ T-cells cells might be acting locally in the gastric mucosa to inhibit pathogenic T-cell responses. Whether *Helicobacter*-activated $CD80^+$ $IL\text{-}10^-$ B-cells (which, in contrast to $IL\text{-}10^+$ B-cells, migrate to the gastric mucosa in large numbers) generate Tr-1 cells in the MLN or directly in the gastric mucosa is currently not known. We and others have shown previously that chronic *Helicobacter* infection induces mucosa-associated lymphoid tissue (MALT) in the gastric mucosa of humans and experimentally infected

mice (24, 27, 37). In MALT, T- and B-cells form organized structures mimicking reactive lymphoid follicles, which could potentially serve as the sites of Tr-1 cell priming.

At least one other mechanism has been described through which B-cells can efficiently induce Treg with suppressive activity (36). Using co-culture models as well as contact hypersensitivity and allogeneic transplant models, Reichardt and colleagues showed that naïve B-cells can prime $CD4^+CD25^+$ regulatory T cells, which in turn potently suppress T-cell responses *in vitro* and *in vivo* (36).

The requirement for TLR-2 expression and MyD88 signaling for a regulatory activity of B-cells and for Tr-1 cell generation is consistent with the greatly enhanced susceptibility of $TLR\text{-}2^{-/-}$ and $MyD88^{-/-}$ animals to *Helicobacter*-induced gastric histopathology. The phenotypes of both strains suggest that TLR-2 is required for priming of the regulatory, but not the effector branches of the adaptive immune response to *Helicobacter* infection. Indeed, effector T-cell activation is not impaired in the $TLR\text{-}2^{-/-}$ strain, which controls bacterial colonization more efficiently than the wild type strain. Our data is consistent with a recent report by Rad *et al*., who identified TLR-2, and to a lesser extent TLR-4 and -9 as essential for the recognition of *Helicobacter* by dendritic cells *in vitro* (38).

In conclusion, we show here that B-cells play an essential role in the suppression of excessive immunopathology driven by *Helicobacter* infection in murine models. We provide evidence for a novel IL-10-independent, but MyD88-dependent, mechanism of immune counter-regulation by B-cells, which generates IL-10-secreting Treg with suppressive activity *in vitro* and *in vivo*. Our data have implications for vaccine development against *Helicobacter*, which must attempt to override immune counter-regulation to achieve sterilizing immunity upon challenge infection (39). The cooperative B-/T-cell-mediated suppression of immunopathological gastric responses to *Helicobacter* infection provides a possible explanation for why a majority of *Helicobacter*-infected individuals never develop overt disease symptoms but are protected from long-term manifestations of *Helicobacter* infection.

Acknowledgements

We would like to thank Iris Hitzler, Isabelle Arnold, Vanessa Craig and Mathias Oertli for experimental assistance and comments on the manuscript. The authors declare no competing financial interests.

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Footnotes

[1]This study was funded by grants from the Swiss National Science foundation, the UBS foundation and the Swiss Cancer League to A.M. Additional funding was supplied by the University Research Priority Program in Systems Biology/Functional Genomics.

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[3]Abbreviations used in this paper: T_H, T helper, Hp, *Helicobacter pylori*; Hf, *Helicobacter felis*; wt, wild type.

Figures and Figure legends

Figure 1

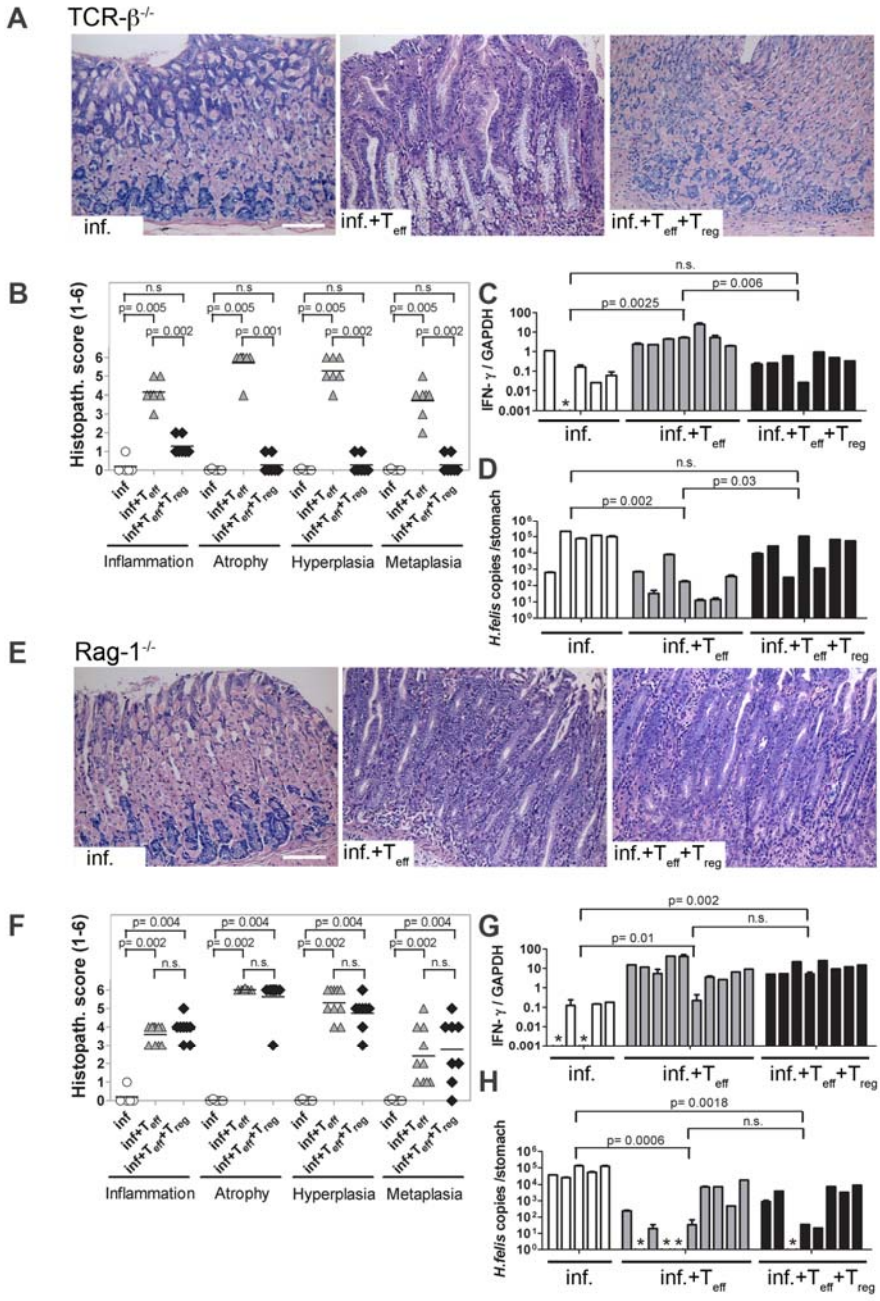


Figure 1: Regulatory T-cells block the gastric histopathology induced by co-transferred effector T-cells in the TCR-β$^{-/-}$, but not the Rag-1$^{-/-}$ background. TCR-β$^{-/-}$ mice (*A-D*) and Rag-1$^{-/-}$ mice (*E-H*) received either 250.000 immunomagnetically isolated CD4^{+}CD25^{-} effector T-cells and 250.000 CD4^{+}CD25^{+} Treg ('T_{eff}+T_{reg}') or effector cells only ('T_{eff}') and were infected with

H. felis on the same day. A control group was infected but did not receive cells ('inf'). All mice were sacrificed four weeks p.i. *A,E,* Representative micrographs of Giemsa-stained sections. The scale bar indicates 50 µm. *B,F,* Histopathology scores assigned to every mouse for all indicated parameters on a scale of 0-6. Horizontal bars indicate the means. *C,G,* Gastric IFN-γ production as assessed by quantitative real time RT-PCR. IFN-γ expression is normalized to GAPDH expression. *D,H, H. felis* colonization as assessed by quantitative PCR of the *flaB* gene using total gastric genomic DNA as template. Vertical bars indicate SEM; asterisks designate values below the detection limit.

Figure 2

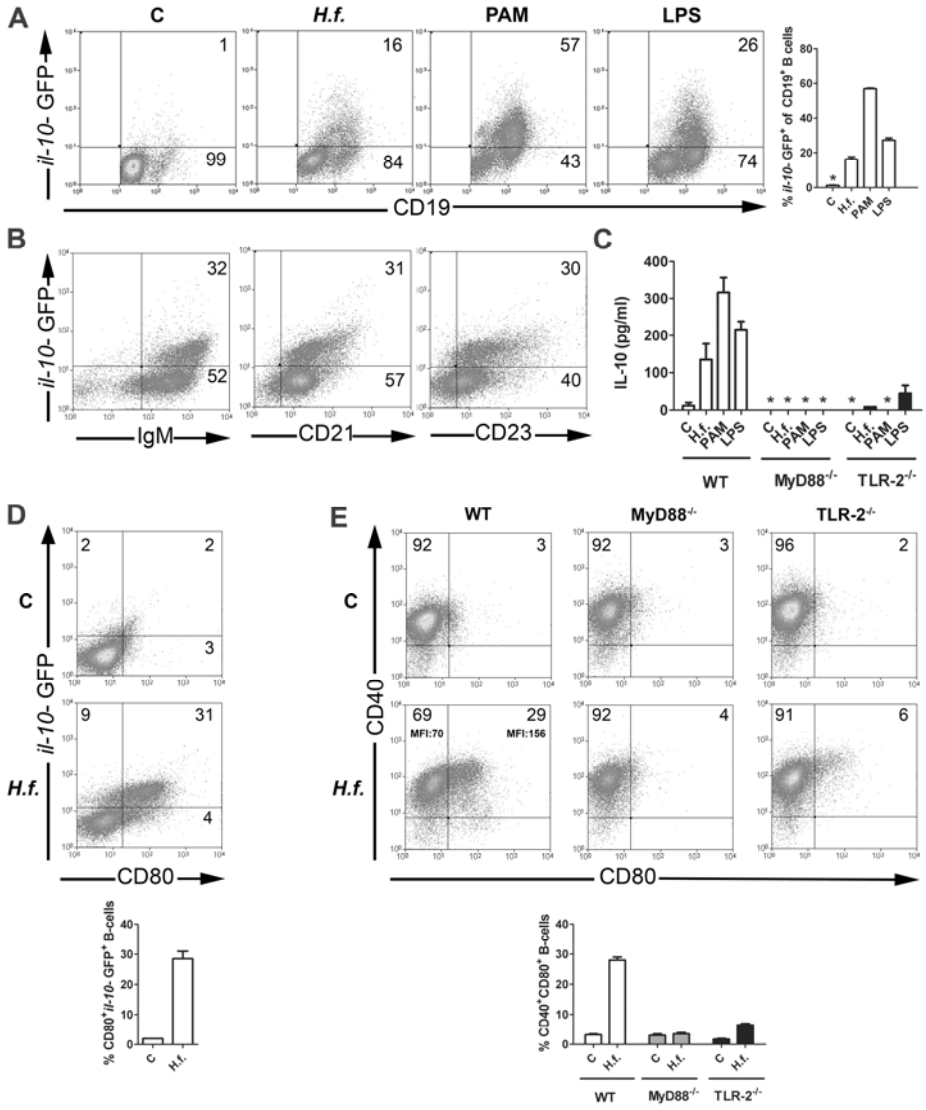


Figure 2: B-cells produce IL-10 and up-regulate CD80 in a MyD88- and TLR-2-dependent manner upon exposure to *Helicobacter* sonicate. B-cells were immunomagnetically isolated from the spleens of *il-10*-GFP knock-in reporter mice (*A,B,D,E*), or wild type, MyD88$^{-/-}$ and TLR-2$^{-/-}$ mice (*C*) and treated for 48 hours with 10µg/ml *Helicobacter* sonicate ('*H.f.*'), 5µg/ml Pam3Cys or 10µg/ml LPS. The cultures were stained for expression of the indicated surface markers (CD19, IgM, CD21, CD23, CD80, CD40) and analyzed for induction of *il-10*-GFP expression. *A*, *Il-10*-GFP expression in the CD19^{+} B-cell population. Representative dot plots and the averages of triplicate cultures are shown in the left and right panels. Values indicate CD19^{+} *il-10*-GFP$^{+/-}$ cells in %; vertical bars indicate SEM. *B*, IgM, CD21 and CD23 expression of the same cultures as shown in A, gated on the CD19^{+} population. *C*, IL-10 expression as

determined by ELISA of wild type, MyD88$^{-/-}$ and TLR-2$^{-/-}$ B-cells treated as indicated. Averages +/- SEM are shown. Asterisks indicate values below the detection limit. *D*, *Il-10*-GFP^{+} B-cells induced by exposure to *H. felis* extract are CD80^{+}. Representative dot plots and averages of triplicate cultures are shown. *E*, *Helicobacter* sonicate induces CD80 and CD40 up-regulation in wild type, but not MyD88$^{-/-}$ and TLR-2$^{-/-}$ B-cells. Representative dot plots and averages of triplicate cultures are shown. MFI stands for mean fluorescence intensity of the CD40 signal in the CD80^{+} and CD80^{-} populations.

Figure 3

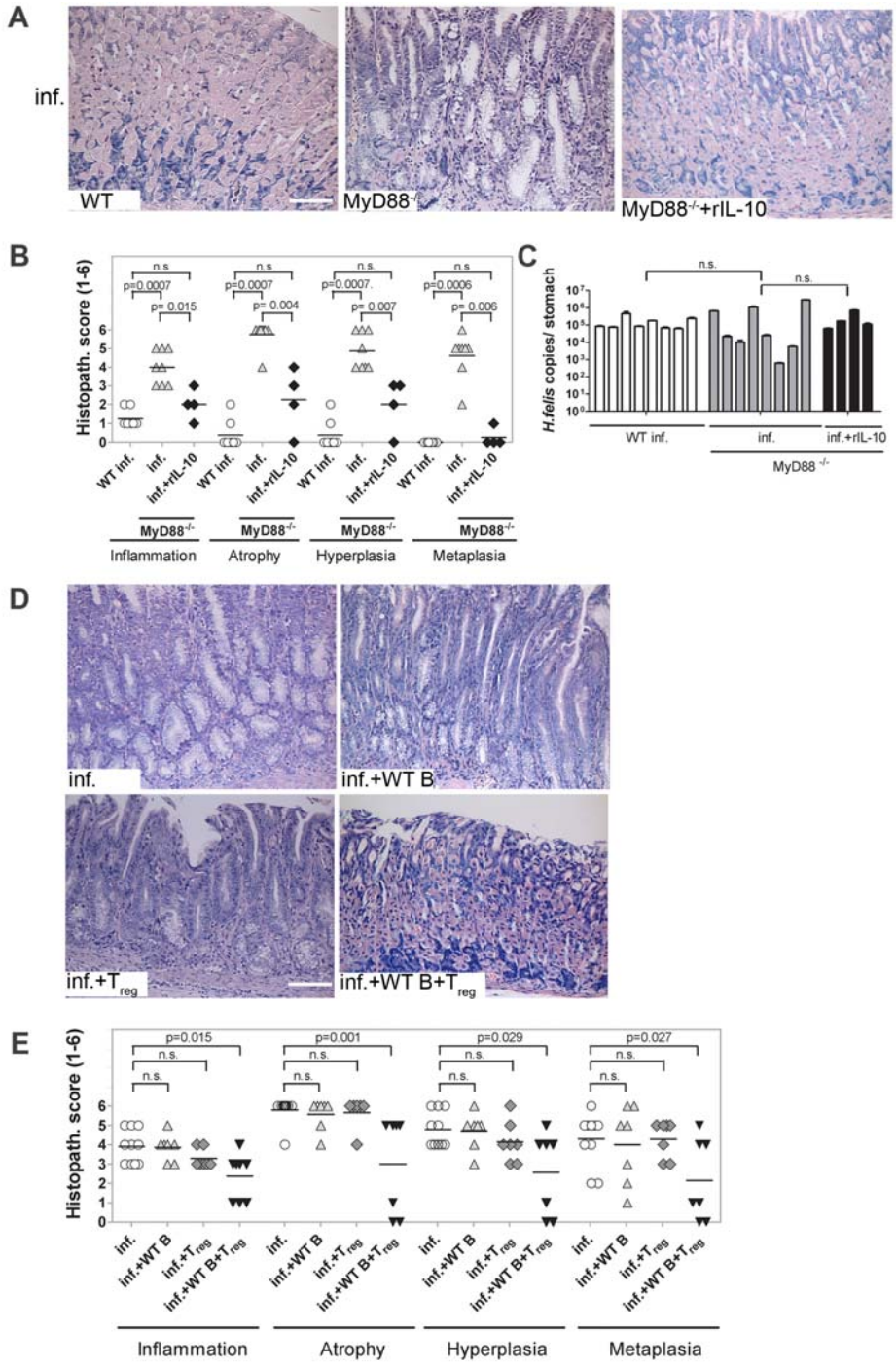


Figure 3: The accelerated gastric histopathology of MyD88$^{-/-}$ mice can be prevented by administration of recombinant IL-10 or by adoptive co-transfer of wild type B-cells and CD4^{+}CD25^{+} splenic Treg. *A,B,C*, Wild type and MyD88$^{-/-}$ mice were infected for one month with *H. felis*. A group of infected MyD88$^{-/-}$ animals further received thrice-weekly intraperitoneal doses

of recombinant murine IL-10 during the entire month. *A,* Representative micrographs of Giemsa-stained sections of one mouse per group. The scale bar indicates 50 µm. *B*, Histopathology scores assigned as described in Figure 1. *C, H. felis* colonization as determined by quantitative PCR of the *flaB* gene. *D,E,* MyD88$^{-/-}$ mice were infected for one month with *H. felis*, and received 250.000 immunomagnetically isolated, splenic B-cells and/or an equal number of splenic CD4^{+}CD25^{+} Treg as indicated. *D*, Representative micrographs. *E*, Histopathology scores of all mice included in the four treatment groups. Horizontal bars indicate mean values.

Figure 4

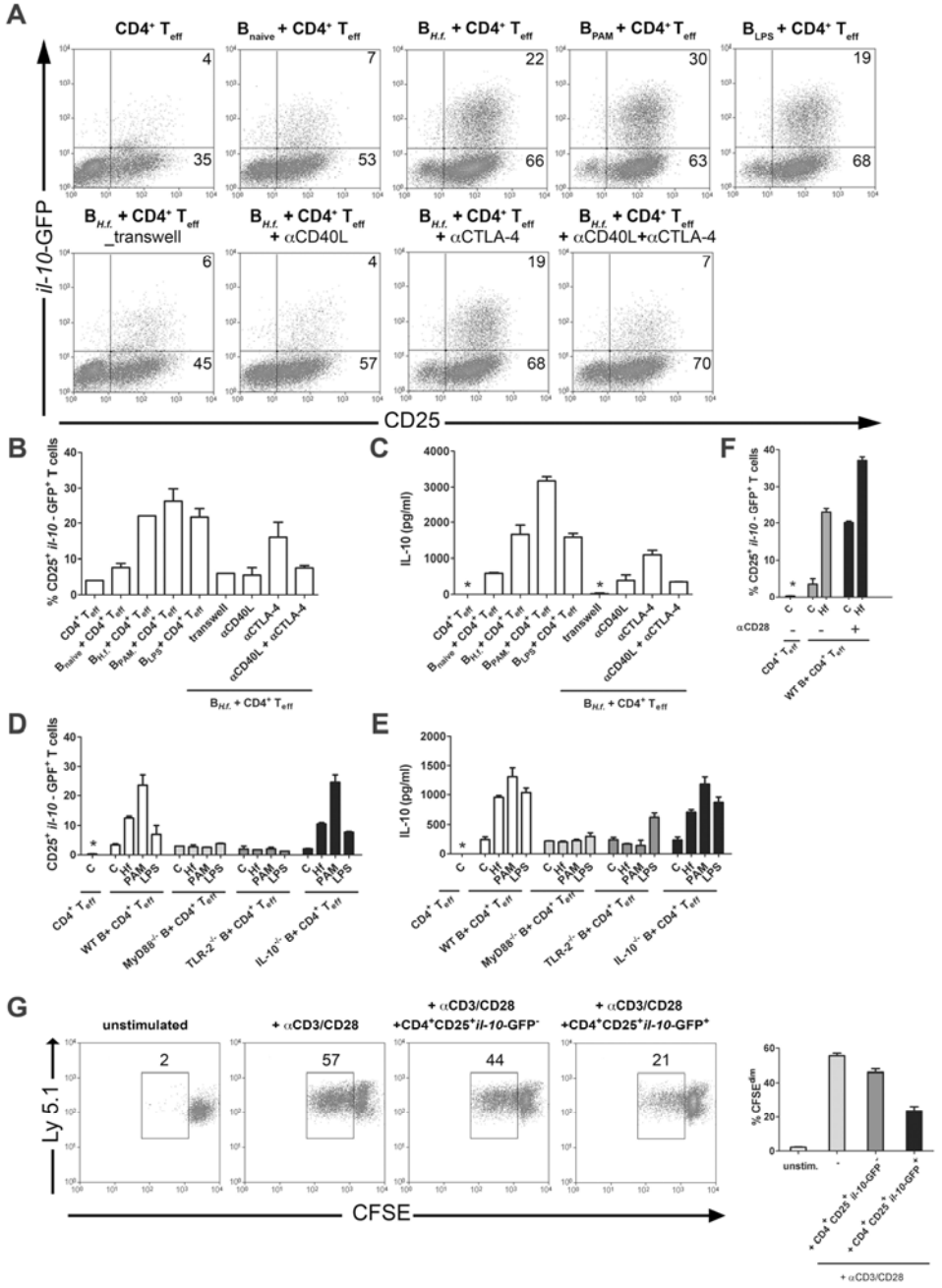


Figure 4: *Helicobacter* extract-pretreated B-cells induce IL-10 expression in conventional CD4$^+$ T-cells in a manner depending on MyD88 and TLR-2 on the part of the B-cell, and on TCR- and CD28 signaling on the part of the T-cell. *A-C,* Wild type splenic B-cells were treated for four hours with 10µg/ml *Helicobacter* sonicate ('*H.f.*'), 5µg/ml Pam3Cys or 10µg/ml LPS prior to co-culturing for four days with *il-10*-GFP knock-in CD4$^+$CD25$^-$ T-cells in the presence of anti-CD3 crosslinking antibody and IL-2. Where indicated, the two populations were separated by a

transwell filter, or anti-CD40L and/or anti-CTLA-4 blocking antibody was added at 10µg/ml final concentration. The co-cultures were stained for CD4 and CD25; all events in the $CD4^+$ gate are plotted. *A*, Representative dot plots of $CD4^+$ T-cells expressing *il-10*-GFP and stained for CD25. The co-culture conditions are indicated above the plots. Values in quadrants indicate *il-10*-GFP^+ $CD25^+$ and *il-10*-GFP^- $CD25^+$ cells in % of all $CD4^+$ cells. *B*, Averages +/- SEM of *il-10*-GFP^+ $CD25^+$ T-cells of triplicate cultures as shown in A. *C,* IL-10 secretion into the culture supernatants as determined by ELISA of the cultures shown in *A* and *B*. *D,E*, Wild type, $MyD88^{-/-}$,$TLR\text{-}2^{-/-}$ and $IL\text{-}10^{-/-}$ splenic B-cells were treated for four hours with 10□g/ml *Helicobacter* sonicate (‘*H.f.*’), 1µg/ml Pam3Cys or 10□g/ml LPS prior to co-culturing for four days with *il-10*-GFP knock-in $CD4^+CD25^-$ T-cells in the presence of anti-CD3 crosslinking antibody and IL-2. *Il-10*-GFP and CD25 expression were analyzed as described in A and B. *D*, Averages +/- SEM of *il-10*-GFP^+ $CD25^+$ cells in % of all $CD4^+$ T-cells of triplicate cultures. *E*, IL-10 production of the cultures shown in *D*. *F*, B- and T-cells were co-cultured as described in A; a CD28-crosslinking antibody was added where indicated at a final concentration of 1µg/ml. *G*, FACS-sorted $CD4^+CD25^+$ *il-10*-GFP^+ and $CD4^+CD25^+$ *il-10*-GFP^- T-cells were added to CFSE-labeled $Ly5.1^+CD4^+CD25^-$ effector T-cells at a 1:1 ratio. The effector cells were stimulated by CD3/CD28 crosslinking, and their proliferation was determined by CFSE dilution. The values indicate $CFSE^{dim}$ cells in % of all $Ly5.1^+$ cells. Representative dot plots are shown in the left panels; averages +/- SEM of $CFSE^{dim}$ cells in % of all $Ly5.1^+$ T-cells are shown in the right panel.

Figure 5

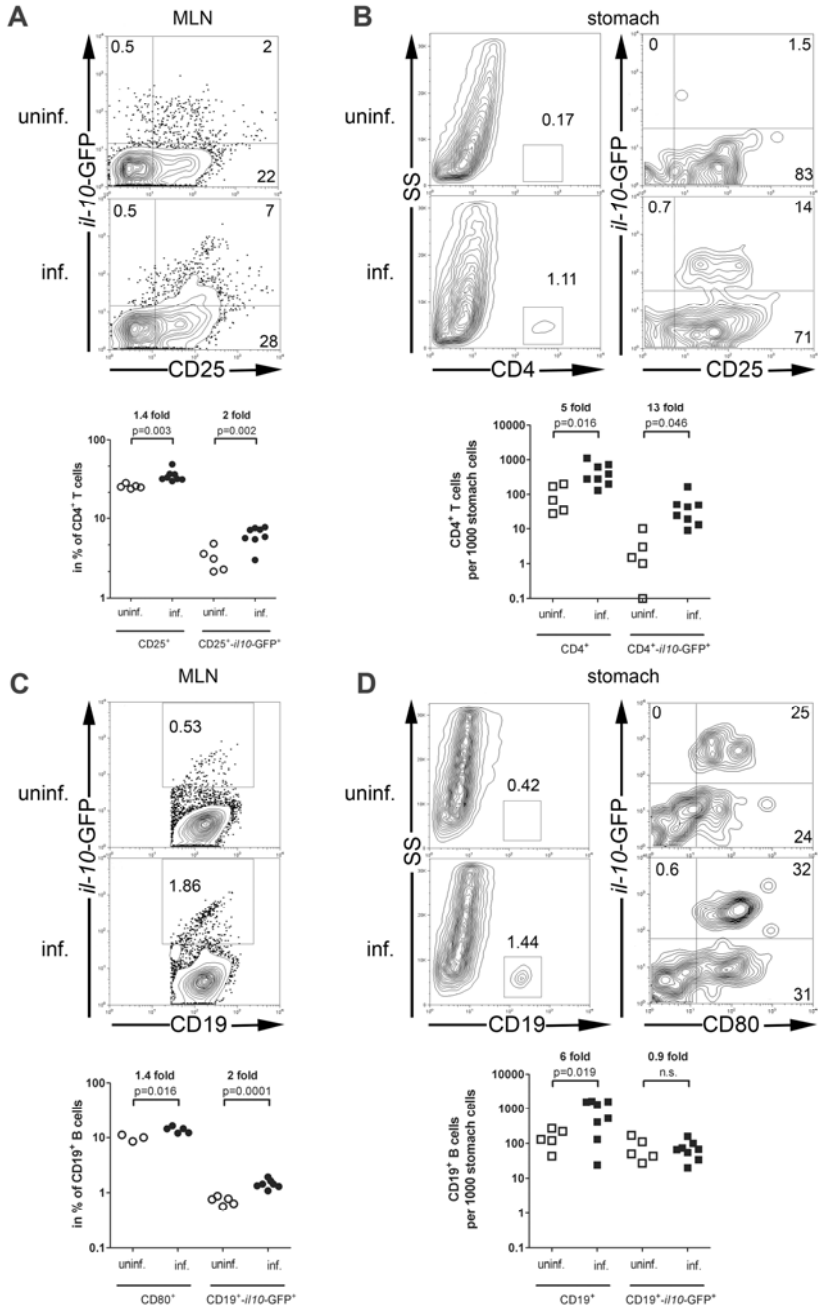


Figure 5: *Il-10*-GFP$^+$ T-cells, but not B-cells, are recruited to the *H. felis*-infected gastric mucosa. Eight *il-10*-GFP knock-in mice were infected for three months and compared to five uninfected controls with respect to *il-10*-GFP expression in the CD4$^+$CD25$^+$ T-cell (*A,B*) and CD19$^+$ B-cell (*C,D*) compartments in the MLN and stomach. *A,B*, MLN (*A*) and stomach (*B*) single cell preparations of every mouse were stained for CD4 and CD25 and analyzed with regard to *il-10*-GFP expression. *C,D,* The same single cell preparation as described in A and B

were stained for CD19 and CD80 and analyzed with regard to *il-10*-GFP expression. CD80 expression data are only available for a subgroup of three uninfected and five infected mice. In all panels, dot plots of representative mice are shown (upper panels) along with the quantification for all five uninfected and eight infected mice per group (lower panel).

Figure 6

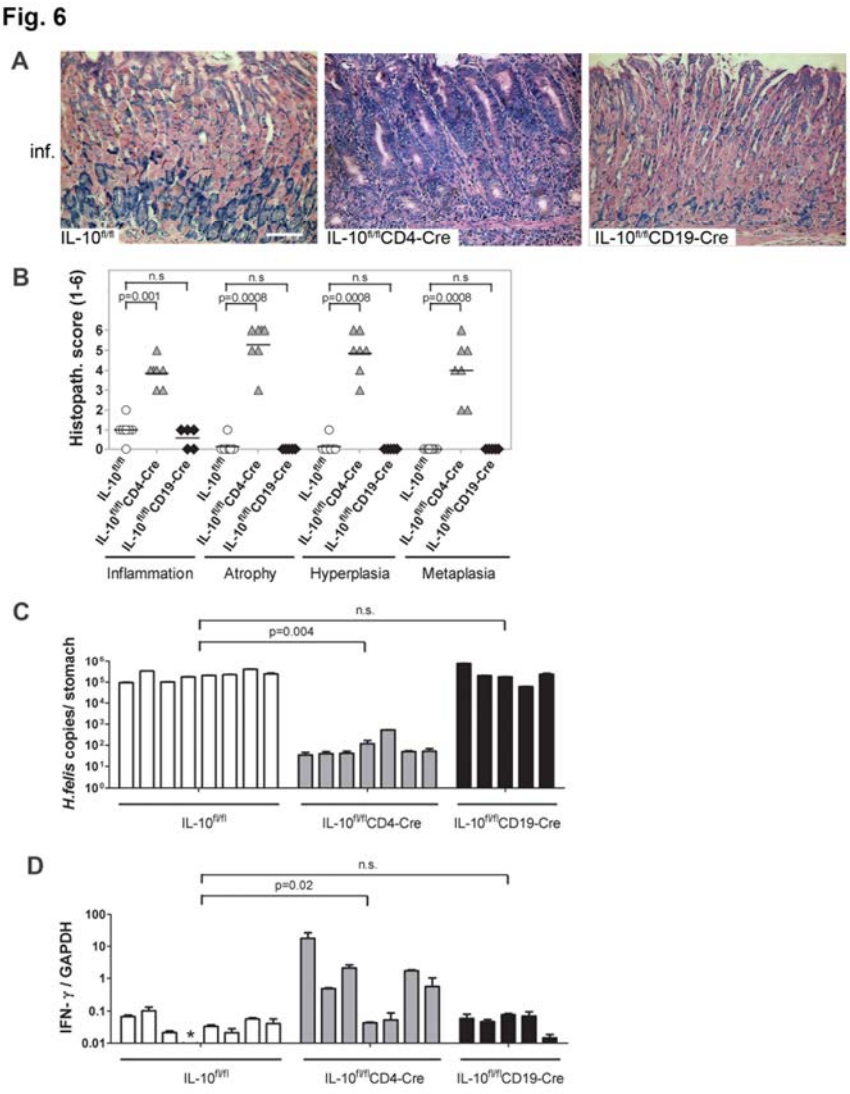


Figure 6: T-cellular, but not B-cellular IL-10 expression is required for suppression of *Helicobacter*-associated gastric histopathology. IL-10$^{fl/fl}$, IL-10$^{fl/fl}$CD4-Cre and IL-10$^{fl/fl}$CD19-Cre mice were infected for one month with *H. felis* and analyzed with respect to gastric histopathology, *H. felis* colonization and gastric IFN-γ expression. *A*, Representative micrographs of Giemsa-stained sections of one mouse per group. The scale bar indicates 50 µm. *B*, Histopathology scores assigned as described in Figure 1. *C*, *H. felis* colonization as determined by quantitative PCR of the *flaB* gene. *D*, Gastric INF-γ expression as assessed by real time RT-PCR analysis. IFN-γ levels are normalized to GAPDH expression.

Figure 7

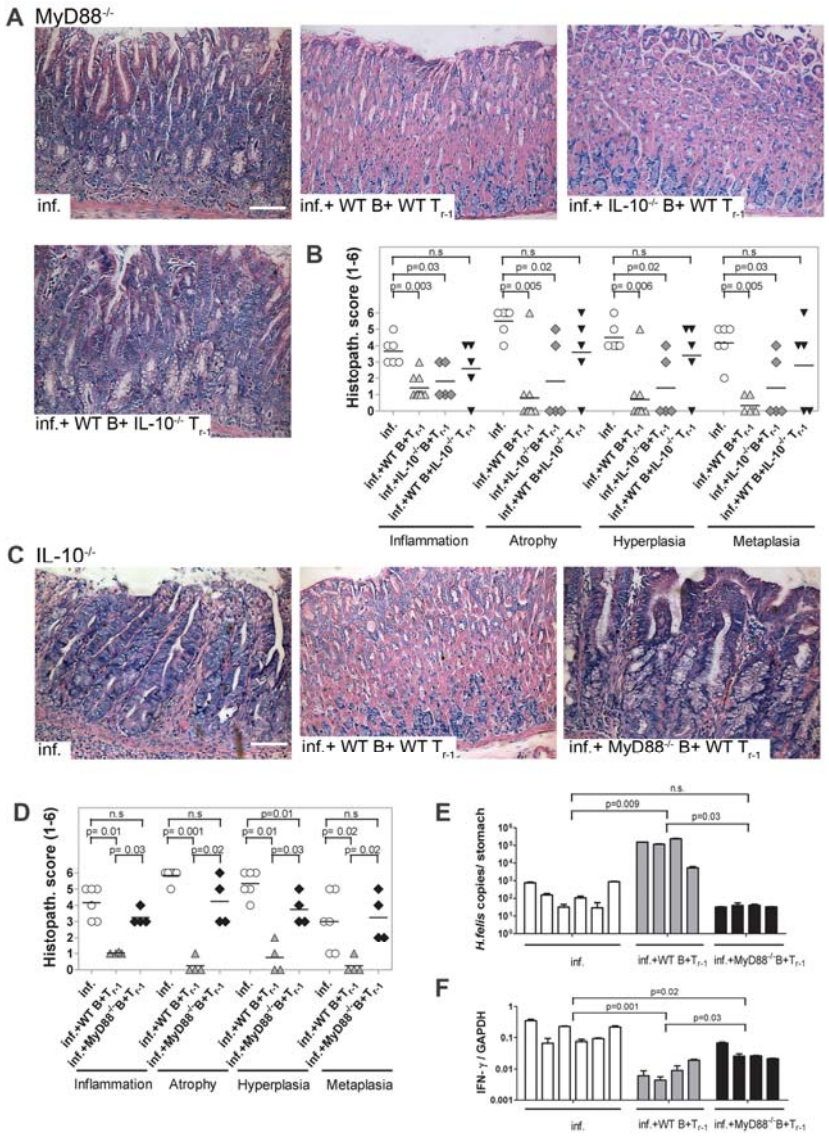
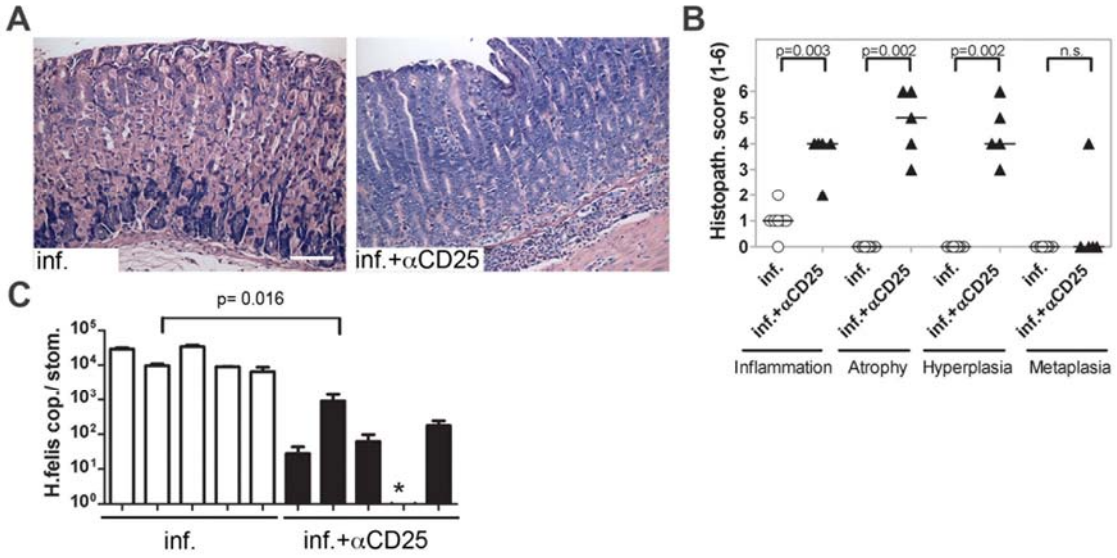


Figure 7: Suppression of gastric pathology in MyD88$^{-/-}$ and IL-10$^{-/-}$ mice requires B-cellular MyD88 signaling and T-cellular IL-10 expression. MyD88$^{-/-}$ (*A,B*) and IL-10$^{-/-}$ (*C-E*) mice were infected with *H. felis* for one month and three weeks, respectively. On the day of infection, the mice received i.v. injections of 250.000 B- and Tr-1 cells (wild type, IL-10$^{-/-}$ or MyD88$^{-/-}$), that were co-cultured for four days at a ratio of 1:1 prior to injection. *A,C*, Representative micrographs of Giemsa-stained sections of one mouse per group. The scale bar indicates 50 µm. *B,D*, Histopathology scores assigned as described in Figure 1. *E, H. felis* colonization as determined by quantitative PCR of the *flaB* gene.

Supplemental Figures 1-4

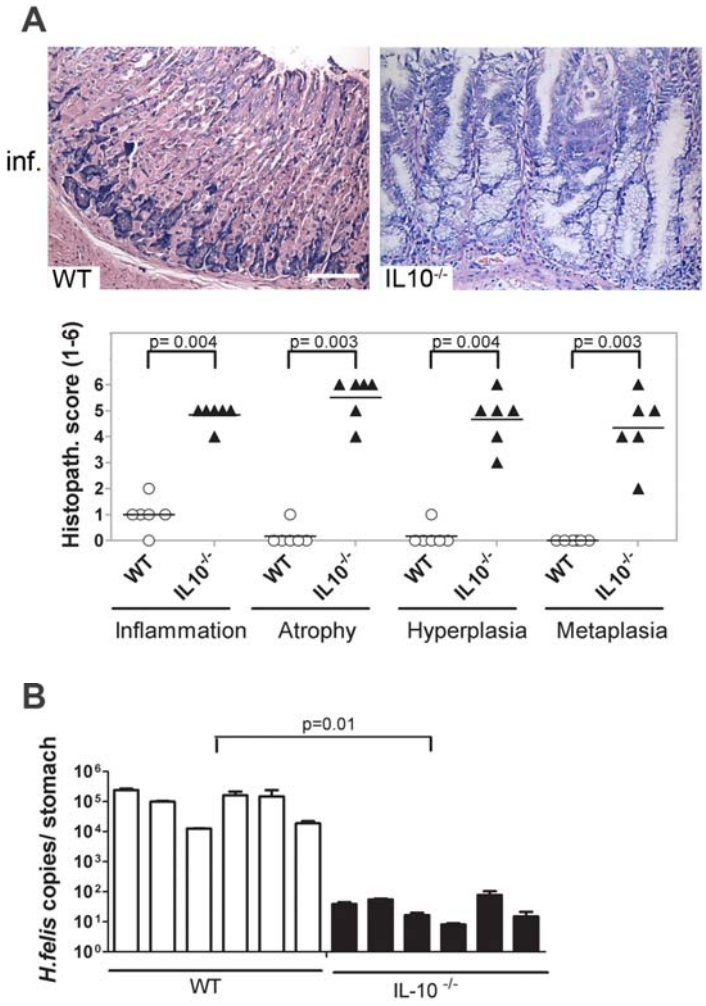
Supplemental Figure 1



Supplemental Figure 1: CD4$^+$CD25$^+$ Treg are essential for the prevention of excessive gastric *Helicobacter*-associated immunopathology.

(A-C) Wild type mice were infected for one month with *H. felis* and received weekly injections of anti-CD25 antibody; a control group was infected, but left untreated. All mice were assessed with respect to gastric histopathology and colonization; representative Giemsa-stained sections are shown in A and histopathology scores are shown in B. The scale bar indicates 50µm. Colonization as determined by *flaB*-specific quantitative PCR on total gastric genomic DNA is shown in C.

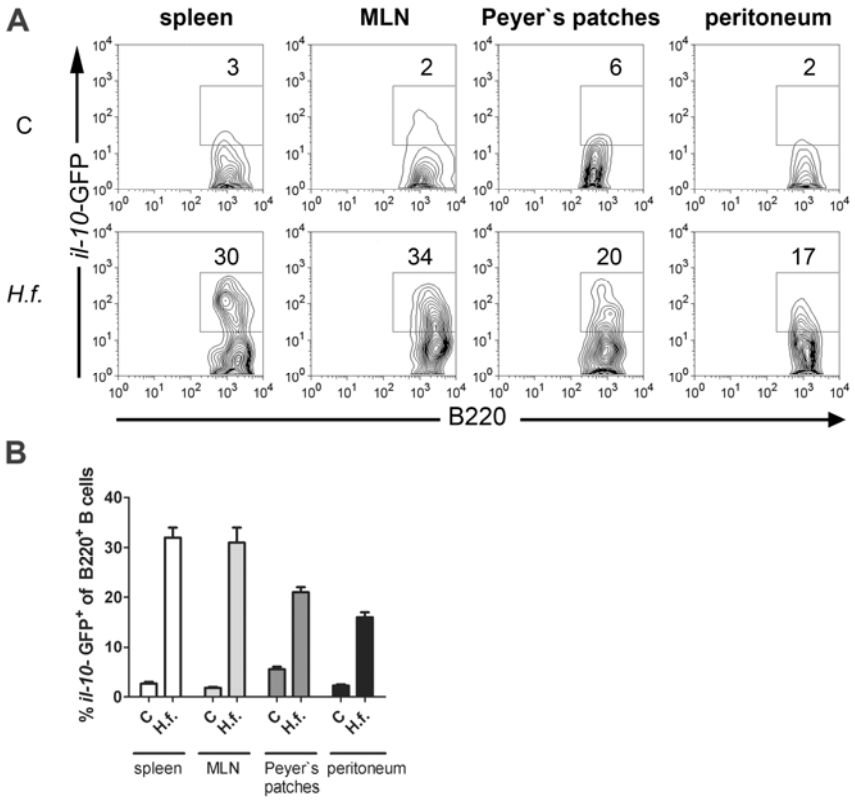
Supplemental Figure 2



Supplemental Figure 2: IL-10$^{-/-}$ mice develop greatly accelerated gastric preneoplastic pathology and efficiently clear *H. felis* infection.

(A,B) Wild type and IL-10-/- mice were infected for one month with *H. felis* and analyzed with respect to gastric histopathology (A) and colonization (B). Representative Giemsa-stained sections and histopathology scores are shown in A; colonization levels as determined by *flaB*-specific quantitative PCR on total gastric genomic DNA are shown in B. The scale bar indicates 50µm.

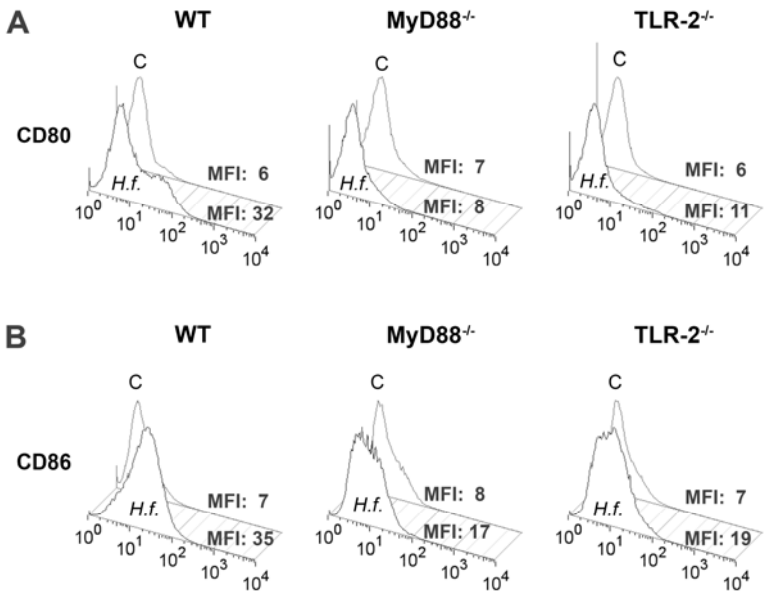
Supplemental Figure 3



Supplemental Figure 3: B-cells from various organs can be induced to express IL-10 by treatment with *H. felis* extract.

(A,B) B-cells were immunomagnetically purified from single cell suspensions of pooled spleens, MLN, Peyer's patches and peritoneum of three mice. The B-cells were treated with *Helicobacter* extract for 72 hours (*'H.f.'*) or left untreated ('C'), and were stained for expression of B220. Representative dot plots of *il-10*-GFP production in the $B220^+$ population are shown in A and averages of triplicate cultures are shown in B.

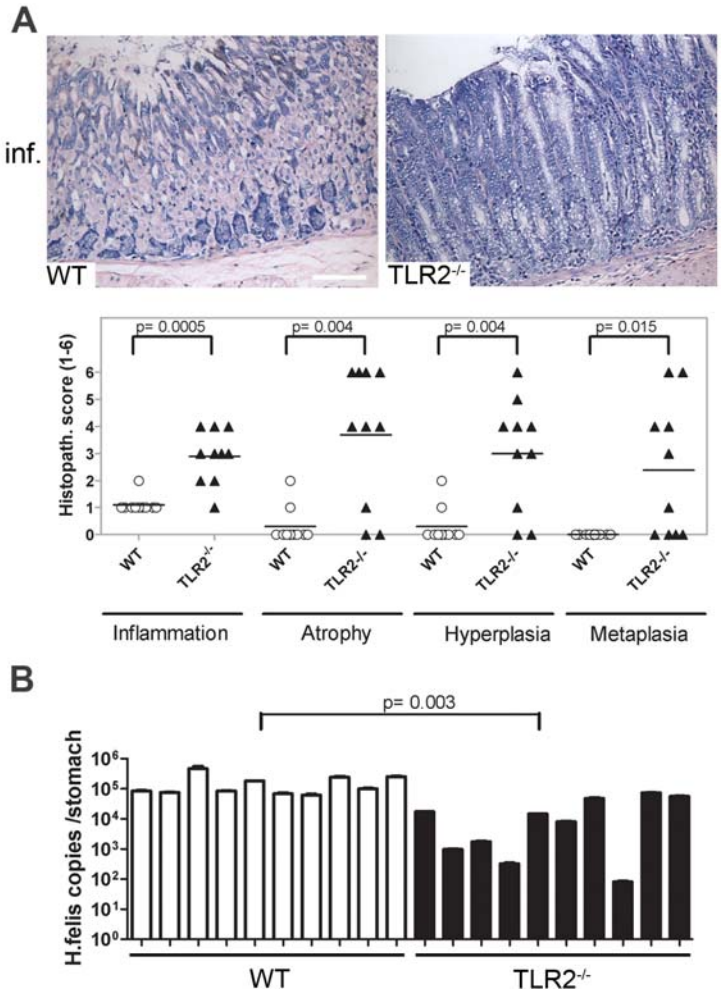
Supplemental Figure 4



Supplemental Figure 4: The *Helicobacter*-induced up-regulation of CD80 and, to a lesser extent, of CD86 on B-cells depends on MyD88 and TLR-2.

(A,B) Splenic B-cells were immunomagnetically purified from wild type, MyD88$^{-/-}$ and TLR-2$^{-/-}$ mice, treated with *Helicobacter* extract for 24 hours (*'H.f.'*) or left untreated ('C'), and stained for expression of CD80 (A) and CD86 (B). MFI indicates mean fluorescence intensity.

Supplemental Figure 5



Supplemental Figure 5: TLR-2$^{-/-}$ mice develop greatly accelerated gastric preneoplastic pathology and efficiently reduce *H. felis* infection.

(A,B) TLR-2$^{-/-}$ mice were infected for one month with *H. felis* and analyzed with regard to gastric histopathology (A) and *H. felis* colonization (B). Representative Giemsa-stained sections and histopathology scores are shown in A; colonization levels as determined by *flaB*-specific quantitative PCR on total gastric genomic DNA are shown in B. The scale bar indicates 50µm.